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Genetic and cytological analyses of four partial-sterile mutants in soybean (Glycine max L. Merr.)

Telma Nair S. Pereira
Iowa State University

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Pereira, Telma Nair S., Ph.D.

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U·M·I
300 N. Zeeb Rd.
Ann Arbor, MI 48106

Genetic and cytological analyses of four partial-sterile mutants in soybean (*Glycine max* L. Merr.)

by

Telma Nair S. Pereira

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I want to dedicate this work to my parents Joao and Maria de Lourdes da Silva Santana. Their help and support throughout my whole life has made all of this possible. Words cannot express my gratitude.

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GENERAL INTRODUCTION

An Explanation of Dissertation Format

This dissertation includes two manuscripts preceded by a general introduction. The first manuscript reports the genetic and cytological studies of soybean partial-sterile mutant 1 (PS-1). The second manuscript reports the genetic and cytological analyses of three partial-sterile mutants, PS-2, PS-3, and PS-4. All four partial-sterile mutants were obtained in a soybean population containing a transposable element. The manuscripts are followed by a general conclusion section. References cited in the general introduction and general conclusions are listed in the general reference section.

Literature Review

Transposable elements

Transposable elements are segments of DNA that possess the ability to move to new locations in the genome. They can move from one chromosome to another or within the same chromosome. Transposable element movement can cause chromosomal rearrangements such as deletions, inversions, duplications and translocations (Federoff, 1983). Transposable elements first were analyzed and described in maize by McClintock (1942, 1949, 1950) but according to Nevers et al.

(1986) at least 20 different mutable loci have been reported in maize and 35 mutable genes in snapdragon and in more than 56 alleles in 31 other plant species. They also have been described in bacteria, yeast, and *Drosophila* (Calos and Miller, 1980). Epperson and Clegg (1992) reported unstable white flower color gene and their derivatives in the morning glory as a result of a transposon insertion.

The transposable element can be simple in structure, consisting of two components, a regulatory or autonomous element and a receptor or nonautonomous element (Federoff, 1983). The regulatory or autonomous element has all components necessary for transposition and the receptor or nonautonomus element cannot move without the presence of a regulatory element in the genome. The receptor element might have lost one or more functions required for transposition but the structural features necessary for transposition remain intact (Pohlman et al., 1984). Most plant transposable elements possess an inverted terminal repeat sequence, and generate a duplication at the target site upon integration (Nevers et al., 1986).

The insertion of transposable element into a locus can interfere with the normal functioning of the locus. It can block transcription of a gene (Goldberg et al., 1983) or the transcripts that are produced encode a defective gene product or no gene product (Gierl et al., 1985). The excision of the

transposable element from the locus also has many consequences. If the excision is precise, that is the element and the target site duplication are removed, normal gene function is restored and, as a result, the wild type DNA is restored. An imprecise excision changes the DNA sequence of a locus (Doring and Starlinger, 1984). Frequently, the excision leaves a portion of the target site duplication in the gene. The extra base pairs that remain after excision may cause frameshift mutations or the addition of one or more amino acids to the protein encoded by the locus. Imprecise excision may also remove extra base pair(s) or change the sequence of the gene, leading to the appearance of new mutant alleles. Usually the mutable phenotype is characterized by variegation, but not all plant variegations are the result of transposable elements (Nevers et al., 1986). Schwarz-Sommer et al. (1985), believe that transposable elements have an important role in the evolution of species.

In soybean several insertion elements (**Tgm**) and three mutable alleles have been identified. The **Tgm** family insertions do not transpose. The first soybean insertion element **Tgm1** was identified as blocking seed lectin (**Le**) expression and it represents a 3.4 kb DNA insertion (Goldberg et al., 1983; Vodkin et al., 1983; Rhodes and Vodkin, 1985). The presence of the insert reduces the levels of **Le** mRNA, causing reduction in the amount of seed lectin. This insertion

has many molecular features of a transposable element such as target site duplication and inverted repeats (Nevers et al., 1986). The DNA sequence of **Tgm1** and the lectin gene showed that the 3 bp sequence of the lectin gene had been duplicated (Vodkin et al., 1983). Other transposable elements, such as **Tam1** in snapdragon and **En** in maize also generated a 3 bp duplication at their insertion site (Bonas et al., 1984; Pereira et al., 1986). Another feature of transposable elements is the presence of terminal inverted repeats. The two arms of **Tgm1** were inverted imperfect repeats. Repetitive palindromic sequences of approximately 54 bp were present in the left arm. The right and left arms contained 2 and 13 hairpin structures, respectively. The molecular proof that **Tgm1** can transpose was not found, but in maize the deletion derivatives of **Ac** (**Ds** elements) either cannot transpose or they transpose only in the presence of a fully functional element (McClintock, 1949; Federoff et al., 1983).

Peterson and Weber (1969) described genetically a soybean mutant that caused leaf variegation (green/yellow). It was observed that the mutation arose at the Y_{18} locus, a nuclear gene that contributes to chloroplast development. The unstable allele gives rise to sectored or variegated green/yellow leaves which indicate the somatic mutability events. The Y_{18}-**m** allele mutates at high rates in both directions at high rates to dominant green Y_{18} and to recessive yellow y_{18} (somatically

and germinally) and also is temperature sensitive (Chandlee et al., 1989; Sheridan and Palmer, 1977). Molecular analysis indicated that the Y_{18}-**m** locus may represent a new and completely independent transposable element system distinct from the **Tgm** family of elements (Chandlee et al., 1989).

A mutation at the **R** locus was identified in soybean. The mutable allele **r-m** conditions a variegated distribution of a black and/or concentric ring of pigmentation superimposed on brown seed coat (Chandlee et al., 1989). Molecular analysis of the **r-m** locus indicated that the mutability of the allele may be associated with rearrangements of **Tgm1**-related sequences in the soybean genome (Chandlee et al., 1989).

Anthocyanin biosynthesis in soybean is dependent on the interaction of several genetic loci, such as W_1, W_3, and W_4 (Palmer and Kilen, 1987). Purple flowers indicate the presence of anthocyanin and require dominant alleles at both W_1 and W_4 loci. Homozygous recessive genotypes at w_1 locus do not produce anthocyanin and have white flowers. Dominance at W_1 locus and recessivity at W_4 locus (W_1-, w_4w_4) causes a great reduction of anthocyanin pigmentation. The intensity of pigmentation in W_1-, w_4w_4 plants depends on the presence of a modifier locus, the W_3 locus. Plants with dilute purple and near-white phenotypes are conditioned by W_1-, W_3-, w_4w_4 and W_1-, w_3w_3, w_4w_4 genotypes respectively. Wild-type purple flowered soybean lines have a W_1W_1, w_3w_3, W_4W_4 genotype (Groose et al., 1988). They reported a

soybean line, the "Asgrow mutable " line or w_4-m (w_4-mutable) that they suspected had a transposable element inserted at the w_4 locus. This line has flowers that are entirely purple, entirely near-white flowers and /or flowers with purple and white sectors.

The w_4-m line was found in a population developed from a two-parent cross. Both parent lines were homozygous for wild type dominant alleles at the w_4 locus, so the genotype of one parent was true breeding for white flower (w_1w_1, w_3w_3, W_4W_4) and the other was true breeding for purple flower (W_1W_1, w_3w_3, W_4W_4). Progenies were advanced in bulk to the F_4 generation and after that single-plant selections were made. At F_5 a single progeny row was selected and threshed to produce the F_6 population from which 60 single plants were selected and threshed individually to produce 60 F_7 progenies. In July 1983 at the Asgrow Seed Company at Stonington, Illinois, it was observed that 4 of the 60 F_7 progenies were segregating for mutable-flowered and wild-type purple-flowered plants. These four sublines constituted the Asgrow Mutable line (Groose et al., 1988). Genetic analyses were done and the results indicated that mutable plants carried an unstable recessive (mutable) allele at the w_4 locus and that a dominant allele of the w_4 locus mutated to the unstable recessive allele in a sector of the germline of the single F_4 plant from which the Asgrow Mutable sublines were descended (Palmer et al., 1989). There is no molecular

evidence to prove that the w_4-**m** allele is due to a transposable element but the w_4-**m** has features that are characteristic of transposable elements in other species; 1) the variegation flower phenotype; 2) mutable allele yields germinal revertants at a rate that varies from 5 to 10% per generation and the revertants are stable; 3) occurrence of pale flower phenotypes; and 4) recovery of new mutants such as chlorophyll-deficient mutants (**CD-1** to **CD-8**), necrotic root mutants (**NR-1** to **NR-3**), partial-sterile mutants (**PS-1** to **PS-4**), and a near-sterility mutant (Palmer et al., 1989).

The partial-sterile mutants (**PS-1** to **PS-4**) that were recovered in a transposon tagging study are the subject of this study. The partial-sterile mutants are characterized by reduced number of seeds per pod due to ovule abortion or very early embryo abortion. We propose to study the genetic, inheritance, linkage and allelism of the four mutants and reproductive cytology of these mutants and compare it to reproductive development in fertile plants.

Female sterility in angiosperms

The life cycle of angiosperms alternates between a diploid sporophyte generation and a haploid gametophyte generation. The sporophyte functions to produce spores, which then develop into gametophytes. The differentiated gametophytes in turn produce either the male gametes (sperm)

or female gametes (egg cells). The male gametophyte (pollen grain or microgametophyte) develops within the anther, whereas the female gametophyte (embryo sac or megagametophyte) is a product of the ovule. Sexual reproduction requires the delivery of the sperm nuclei, via the pollen tube, to the embryo sac, where fertilization occurs and the new diploid sporophyte is initiated (Reiser and Fischer, 1993). Abnormalities in either the sporophytic or the gametophytic generations can lead to plant sterility.

According to Gottschalk and Kaul (1974), four different groups of genes control the fertility of higher plants. The asynapsis genes (**as-genes**) cause failure of homologous chromosomes to pair during the early stages of the first meiotic prophase. The desynapsis genes (**ds**-genes) control chiasma frequency or prevent chiasma formation. Both of these gene groups act principally in a similar way, both in micro- and megasporogenesis. They cite a third type, **ms**-genes that act only during microsporogenesis, causing only male sterility. The fourth group are called structural genes that are not related to meiotic behavior but induce a misdifferentiation of the sex organs. The sterility can be due to the failure of ovule differentiation, formation of open carpels or strong reduction or diminution of the sex organs under the influence of the mutated gene. According to the same authors, it is common for a species to have numerous loci

capable of producing male-sterile mutants, however male-fertile, female-sterile mutations are rarely reported in the plant world.

Female sterility in angiosperms has been reported to be associated with a variety of factors including the lack of fertilization, failure of embryo sac to complete its development, high temperatures, reduction of cell divisions in the nucellus, competition due to heavy crop load, suppression of other ovules in a given ovary by the first one to be fertilized, and blockage of the vascular trace leading to the ovule (Mogensen, 1982).

Quercus (Fagaceae), originally had ovaries with six ovules and that typically five of them aborted very early, resulting in the one-seeded acorn at maturity. The occurrence of four different types of abortive ovules has been reported for three species of *Quercus*. Forty-five percent of aborted ovule were due to lack of fertilization; the aborted ovule had both synergids full and intact throughout the life of the ovule and the egg collapsed very early. Ovule abortions also were due to zygote or embryo failure, absence of an embryo sac or empty embryo sac. All types of abortive ovules can occur in the same ovary and it is proposed that all of the ovules that develop in a normal embryo sac are potential seeds, but the first one to be fertilized suppresses the normal development of the others (Mogensen, 1975).

In safflower, the interaction of three unlinked genes are responsible for female sterility. The sterile plants formed normal ovules, but apparently delayed initiation of meiosis, which was subsequently arrested at metaphase I, consequently lead to suppression of the embryo sac (Carapetian and Rupert, 1989). In tomato two types of ovules are reported in unfruitful plants. In one of them, called collapsed type, the ovules develop normally until four megaspores are produced. Thereafter the megagametophyte may degenerate in various intermediate stages of development. In other type, called substitution type, the megaspore mother cell never differentiates nor is there any semblance of normal development of a gametophyte (Rick, 1946).

Female sterility has been reported in *Stylidium* due to recessive lethals factor that induces ovule or seed abortion after entry of the pollen tube into the micropyle of the ovule (Burbidge and James, 1991). In alfalfa, female sterility is due to incomplete development of the integuments that left the nucellus and female gametophyte unprotected and resulted in their destruction (Bingham and Hawkins-Pfeiffer, 1984).

Belling (1914), working with Velvet bean (*Stizolium*), described a heritable type of sterility involving abortion of 50% the pollen grains and embryo sacs, which he called semi-sterility. Blakeslee and Cartledge (1926) also reported semi-sterility in *Datura*. In maize, Singleton and Mangelsdorf

(1940), Clark (1942) and Nelson and Clary (1952) reported three different lethal factors, **lo**, **lo1** and **lo2** respectively. These factors are different but act in a similar way, that is, ovules carrying the lethal factor abort before fertilization and are rarely capable of being fertilized. These factors have a special feature: they do not affect the pollen grains and are not transmitted through the female parent.

Angiospermous plants have yet a third life stage, the endosperm. The endosperm is the product of a second fertilization event, in which the two nuclei (polar nuclei) of female gametophyte's central cell, which is binucleate in most angiosperms, fuses with the second sperm nucleus. The central cell is called endosperm after it divides to become a tissue that provides nutrition to the growing embryo (Chasan and Walbot, 1993).

Thirty-two recessive embryo-lethal mutants of *Arabidopsis thaliana* were isolated by treating mature seeds with an aqueous solution of ethyl methanesulfonate (EMS). Developmental arrest of mutant embryos ranged from the zygote stage to the linear and curled cotyledon stages (Meinke and Sussex, 1979; Meinke, 1982 and 1985; Marsden and Meinke,1985). The defective kernel (**dek**) mutants and embryo-specific (**emb**) mutants in maize also are abnormal in embryonic morphogenesis as well as in the formation of their endosperm. The **emb** mutants can be divided into three groups: a) mutants that

suffered failure in development of the embryo proper and concomitant enlargement of the suspensor; b) mutants that were blocked during the period of the establishment of the embryonic axis; c) mutants blocked during the period of elaboration of embryonic structures (Neuffer and Sheridan, 1980; Clark and Sheridan, 1986, 1988, and 1991; Dolfini and Sparvoli, 1988).

In soybean, sterility-inducting mutations are of two types, male-sterile, female-fertile (MS-FF) mutations and male-sterile, female-sterile (MS-FS); the former eliminate the male reproductive function, the latter eliminate both male and female reproductive function. Also, all known male-sterile mutants in soybean are of the genetic type (Graybosch and Palmer, 1988).

Two structural sterile mutants were reported in soybean. The flower-transformed (**ft**) mutant was obtained by irradiation with gamma rays and the mutant resulted in nondehiscent anthers (Singh and Jha, 1978). The flower-structure (**fs**) mutant has abnormal filament elongation. Self pollination is prevented by the spatial separation of the stigma and anthers. Observations of the gynoecium of mutant flowers revealed some abnormalities such as the failure of the outer integument to form the micropyle; many ovules were positioned abnormally such as ovules appressed either to the ovary wall or to each other; even though with these abnormalities megasporogenesis

and megagametogenesis were normal (Johns and Palmer, 1982). Recently, Skorupska et al. (1993) reported a spontaneous mutation in soybean that alters flower development and produces apetalous male-sterile flowers. They also observed that the gynoecia were characterized by enlarged unfused ovaries and exposed ovules.

Palmer et al. (1992) reported that the ms_1 mutation in soybean also affected the female function. This mutant is associated with reduced female fertility and with increased frequencies of polyembryony, polyploidy and haploidy in the progeny of sterile plants. The failure of postmeiotic wall formation allows up to four megaspore nuclei to undergo megagametogenesis resulting in mature megagametophytes with increased gametophytic cells. Nuclear fusion also occurs during the early stages of megagametogenesis and may result in gametophytic cells of increased ploidy. The multinucleate ms_1 megagametophytes are overcrowded and often lead to abortion, partially explaining the reduced fertility of male-sterile plants (Kennell and Horner, 1985).

Benavente et al. (1989) described the cytology of the cv. KS soybean mutant which is a male-sterile, female-sterile synaptic mutant. The ovules from sterile plants showed various abnormalities. The most consistent abnormality was the failure of the embryo sac to attain the normal size. Small megasporocytes of irregular shape were seen; no linear tetrads

were observed. A range from zero to twenty-eight cells and nuclei of various sizes, were identifiable in small megagametophytes and embryo sacs. Degeneration of these nuclei and cells was observed at the four-nucleate gametophyte stage; ovules contained degenerated nucellar centers without embryo sacs; late postpollination stages were marked by shrunken nucellus and integuments. Degeneration of the gametophyte and embryo sac contents at many developmental stages indicated a wide array of effects, possibly resulting from meiotic irregularities similar to those seen in microsporogenesis of this mutant.

Megasporogenesis and megagametogenesis in soybean (*Glycine max;* Leguminosae)

The soybean ovules are bitegmic, crassinucellate, and campylotropous (Prakash and Chan, 1976). Megasporogenesis, that is the formation of the megaspore, is initiated in the nucellus of very young ovules.

The cells of an ovule primordium are all the same size and one or two days after ovule initiation, several hypodermal archesporial cells are easily distinguishable because they are larger than the neighboring cells and have a more densely stained cytoplasm. One of these cells surpasses the others in size and becomes the functional megasporocyte, called megaspore mother cell, MMC (Carlson and Lersten, 1987).

The megaspore mother cell continues to enlarge and undergoes meiosis approximately 10 days before flowering (Pamplin, 1963). After the second division of meiosis, there is a linear tetrad of haploid megaspores. At this time the outer integument overreaches the inner integument and reaches the nucellar apex (Rembert, 1977). The chalazal megaspore, the furthest from the micropyle, continues to enlarge and the three micropylar megaspores start to degenerate (Carlson and Lersten, 1987). This chalazal megaspore, called functional megaspore, undergoes two mitotic divisions to produce a four-nucleate megagametophyte. The nuclei are positioned at the opposite ends of the embryo sac by the formation of a large vacuole. A third mitosis results in an eight-nucleate megagametophyte with four nuclei located at the chalazal end and four at the micropylar end of the embryo sac (Carlson and Lersten, 1987; Kennell and Horner, 1985). One nucleus from each end migrates to the center of the central cell and subsequently some cell walls are formed, resulting in six uninucleate cells and one binucleate cell with two polar nuclei. The resulting megagametophyte or embryo sac is constituted by the egg apparatus at the micropylar end, antipodal cells at the chalazal end, and the two polar nuclei at the center. The egg apparatus is formed by two synergids and the egg cell. The two synergids have a vacuole at chalazal end with the nucleus toward the micropylar end. The antipodals

disintegrate during the maturation of the embryo sac and at the time of fertilization they are completely degenerated (Carlson and Lersten, 1987; Kennell and Horner, 1985). The two polar nuclei fuse before fertilization to form a single large diploid secondary nucleus within the large central cell and in close proximity to the egg apparatus (Carlson and Lersten, 1987). At time of fertilization the embryo sac has two synergids, an egg cell and a secondary nucleus. One of the synergids disintegrates at fertilization and the other persists until the time of the first division of the zygote (Chamberlin et al., 1993). From the apex of the degenerated synergid is a trail of osmiophilic material that extends to the apex of the zygote and to the secondary nucleus. This trail is the same density as that of the degenerated synergid, suggesting that it is the path of the pollen tube (Chamberlin et al., 1993). At zygote stage the central cell is filled with numerous osmiophilic bodies and large amyloplasts filled with starch. Starch is initiated at the eight-nucleate stage and peaks immediately after fertilization. The zygote shows a distinct polarization of its cytoplasm. The two pockets of cytoplasm are separated by a large vacuole. The zygote starts to divide two to three days after fertilization. Endosperm is cellular (Kennell and Horner, 1985; Chamberlin et al., 1993). At proembryo stage the most obvious change within the central cell is the expansion of the large vacuole displacing the free

nuclear endosperm and starch aggregates to the periphery. With the increase of free nuclear endosperm, the starch reserves decline. By five or six days after fertilization, during the proembryo stage, the starch is gone. The globular stage is characterized by the presence of the embryo proper and the suspensor. This stage can be found in ovules about eight days after fertilization. At this stage and before the cotyledons are initiated, a thin cuticle forms over the surface of the globular head of the embryo proper. Cellularization of the endosperm begins after the development of the globular embryo. The embryo is completely mature 25 days after fertilization, showing both apical and radicle apex (Chamberlin et al., 1993).

GENETIC AND CYTOLOGICAL ANALYSES OF A PARTIAL-STERILE MUTANT (PS-1) IN SOYBEAN (*Glycine max*; Leguminosae)

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Telma Nair S. Pereira, Nels Lersten and Reid G. Palmer

ABSTRACT

Soybean partial-sterile mutant 1 (PS-1) was recovered from a transposon tagging study. The objectives were to study the inheritance, linkage, allelism, and reproductive biology of PS-1 mutant. For inheritance and linkage tests, PS-1 was crossed to Harosoy-w_4, and to chlorophyll-deficient mutant, CD-1, also recovered from the tagging study. For allelism tests, reciprocal crosses were made with PS-1 and three other partial-sterile mutants (PS-2, PS-3, and PS-4) recovered from the same transposon tagging study. Linkage results indicated that the gene for partial sterility in PS-1 is not linked either to the w_4 locus or to the CD-1 locus. The PS-1 mutant is a single-gene recessive and is inherited in a 3:1 ratio. Allelism tests showed that the gene in PS-1 is nonallelic to the gene in PS-2, PS-3, and PS-4. Investigations of microsporogenesis and pollen staining indicated no differences in morphology, stainability or fluorescence between normal and partial-sterile genotypes. The PS-1 mutant is male fertile. Megagametogenesis indicated that early embryo abortion in PS-1

is due to abnormalities, nonmigration and orientation, associated with polar nuclei/secondary endosperm nucleus. The nonmigration prevents the fertilization of the secondary endosperm nucleus with the sperm cell. The failure of double fertilization, absence of endosperm development, and lack of nutrients leads to early embryo abortion.

Key Words: partial-sterile, embryo abortion, transposable element

INTRODUCTION

Transposable elements are segments of DNA that possess the ability to move to new locations in the genome. At the molecular level, the insertion of a transposable element results in a duplication of the target site. The excision of the transposable element removes its DNA sequence from the insertion site. During excision, the target site duplication can undergo a number of changes. As a result, the movement of a transposable element can generate mutations or chromosomal rearrangements that can affect the expression of other genes. Deletions, duplications, translocations and inversions are some of many chromosomal rearrangements that can be produced by chromosome breakage (Saedler and Nevers, 1985; Nevers, Shepherd, and Saedler, 1986).

In 1983, an unstable mutation for anthocyanin

pigmentation in soybean was identified (Groose, Weigelt, and Palmer, 1988). The population containing the mutable allele is called w_4-mutable line. Mutable plants produce both near white and purple flowers, as well as flowers of mutable phenotype with purple sectors on near-white petals (Groose, Schulte, and Palmer, 1990). Mutability is conditioned by a mutable allele at the w_4 locus that is recessive to wild type (Palmer et al., 1989).

According to Groose, Schulte, and Palmer (1990), the allele w_4-m exhibits many features typical of an allele controlled by a transposable element, such as i) variation in reversion frequency; ii) loss of mutability; iii) partial reversion to "pale" alleles; iv) recovery of new mutants.

To provide evidence for transposition, Palmer et al. (1989) conducted a transposing tagging study to recover new mutants. Several were isolated, such as mutants for chlorophyll-deficiency (CD-1 to CD-8), mutants for root necrosis (NR-1 to NR-3), mutants for partial sterility (PS-1 to PS-4), and a mutant for near sterility. The CD-1, CD-2, CD-3, and necrotic roots mutants are inherited independently of the w_4 locus (Palmer, et al. 1989).

Partial-sterile 1 (PS-1) soybean mutant was recovered from an F_{11} family which was descended from a single F_9 plant of the Asgrow Mutable line. The F_{11} family was constituted of 12 plants and all them were partial-sterile (Groose and Palmer,

1987). The pods of partial-sterile plants had a reduced number of seeds per pod and plants were easy to identify at maturity by the high number of one- and two- seeded pods. The reduction in number of seeds per pod could be the result of ovule abortion or very early embryo abortion.

In soybean, sterility-inducing mutations generally are of two types:i) male-sterile, female-fertile (MS-FF); ii) male-sterile, female-sterile (MS-FS). The MS-FF mutations selectively eliminate male reproductive function, but do not greatly affect female reproductive function. In contrast, MS-FS mutations affect both male and female reproduction (Graybosch and Palmer, 1988).

In soybean, **st2** and **st3** mutants are asynaptic sterile genes (Palmer, 1974), **st4** and **st5** are desynaptic steriles (Palmer and Kaul, 1983) and they all are highly male and female sterile(MS-FS). The **ms1** to **ms6** mutants are MS-FF mutants. Female fertility is normal in all mutants except the **ms1** mutant, which has slightly reduced female fertility (Palmer et al., 1992). There are structural genes such as **ft**, flower-transformed mutant (Singh and Jha, 1978) and **fs1 fs2**, flower-structure mutant (Johns and Palmer, 1982). The **ft** mutant produces fertile pollen but the plants are male-sterile due to poor anther dehiscence. The **fs1 fs2** mutants have stamen filaments that fail to elongate normally (Johns and Palmer, 1982). Skorupska, Desamero, and Palmer (1993) reported a

spontaneous mutant in soybean that alters flower development and produces apetalous male-sterile flowers. Partial male sterility is due to incomplete expression of male fertility. Stelly and Palmer (1982) reported that in the **msp** mutant abnormalities in the tapetum lead to the degeneration of sporogenous tissue and that tapetal cells tend to precede abnormalities of associated sporogenous tissue. Partial male sterility also was detected in **ms4** and is suspected for **ms1** and **ms5** (Graybosch and Palmer, 1988).

The objectives of this study were i) to determine if the gene for partial sterility in PS-1 mutant is linked to the w_4 locus and to the chlorophyll-deficient mutant, CD-1; ii) to determine if the gene in PS-1 is an alternative form of the gene in partial-sterile mutants PS-2, PS-3, and PS-4 or if it is a new gene; iii) to study the reproductive cytology of the PS-1 mutant and compare it to reproductive development in fertile plants.

MATERIALS AND METHODS

Genetic Study

Linkage Tests The genetic materials used in this study were partial-sterile soybean mutant, PS-1, chlorophyll-deficient mutant, CD-1, and flower color isoline mutant w_4 in the cultivar Harosoy. PS-1, and CD-1 mutants were found in a transposon tagging study (Groose and Palmer, 1987). The CD-1

is chlorophyll-deficient mutant, which segregates for green and yellow-green plants in a 3:1 ratio. The yellow-green foliage plants have reduced vigor.

Seeds from PS-1, CD-1, and Harosoy-w_4 were sown in summer 1990 and 1991 at the Bruner Farm near Ames, Iowa. At flowering, PS-1 was crossed with Harosoy-w_4, and CD-1. Harosoy-w_4 was always used as the female parent because it has white flowers, a recessive trait that can be used as a morphological marker. The F_1 seed were used to generate the F_2 generation.

In the F_2 generation the number of purple/white fertile and purple/white partial-sterile plants were recorded to estimate the linkage between PS-1 and Harosoy-w_4. The linkage estimation was calculated using the Linkage-1 computer program (Suiter, Wendel, and Case, 1983), which uses the maximum likelihood method.

Fertile and partial-sterile F_2 plants were threshed individually in 1991, and 1992. These $F_{2:3}$ progenies were evaluated each succeeding year, and data were recorded for number of segregating and non-segregating progenies. The χ^2 test was calculated to see if the observed data fit the expected ratio.

The yellow-green F_2 green foliage plants of CD-1 were weak plants and set too few pods to be classified as fertile or partial-sterile. Individual F_2 green foliage plants were threshed and evaluated as $F_{2:3}$ progenies.

Allelism Tests PS-1 is a true breeding mutant for reduced number of seed per pod and PS-2, PS-3, and PS-4 mutants segregate for normal and reduced number of seeds per pod in a 1:1 ratio.

Seeds from PS-1, PS-2, PS-3, and PS-4 were sown in summer 1990 and 1991 at Bruner Farm near Ames. At flowering, five plants in each row of PS's mutants were chosen at random and tagged. Reciprocal cross pollination for allelism tests were made among PS-1 and PS-2, PS-3, and PS-4. The PS-1 to PS-4 tagged plants were classified at maturity for fertility/sterility due to the reduced number of seeds per pod. The F_1 seeds were used to generate F_2 generation; the F_2 seeds to generate the $F_{2:3}$ generation. The χ^2 test was calculated to see if the observed data fit the expected ratio.

Cytological Study

For cytological studies, flower buds at different stages were collected from plants grown in the greenhouse and growth chamber. At growth chamber the temperature and light were controlled. The temperature was 29 C during day and 26 C during night; the photoperiod was 18 hours during four weeks, 16 hours during one week, and 14 hours until maturity.

Most of the serial sections were observed on a Zeiss Standard WL microscope. Photomicrographs were taken with a Contax Data Back Quartz D-5 attachment, using Kodak Technical

Pan film.

Male Gametophyte Several techniques were used to evaluate pollen grains of partial-sterile plants of PS-1 mutant . We started with the simplest methodology and then it became necessary to use more sophisticated techniques.

Microsporogenesis For microsporogenesis, paraffin serial sections were made. Flower buds of various sizes were collected and fixed in cold FAA. After fixation these buds were dehydrated through a graded ethanol/xylene series and infiltrated with Paraplast paraffin over a minimum period of 3 days. Longitudinal and sagittal sections were cut on a rotary microtome at 10 μm, stained with safranin, and overstained with fast green.

Pollen viability Pollen samples were collected from each PS-1 tagged plant for pollen viability/fertility estimation. Pollen samples were collected at anthesis and stored in 70% ethanol at 4 C. Pollen grains were classified as normal/viable or abnormal/inviable based on the staining reaction of mature pollen grains to an iodine potassium iodide, I_2KI, solution. At the same time, measurements of pollen grain diameter were made using a 10X millimetric eyepiece.

A differential staining solution also was used to

distinguish viable from nonviable pollen grains. The differential staining solution (Alexander, 1969) was made by mixing the following :

95% alcohol - 10 ml

Malachite green - 1 ml (1% solution in 95% alcohol)

Distilled water - 50 ml

Glycerol - 25 ml

Phenol - 5 g

Chloral hydrate - 5 g

Acid fuchsin - 5 ml (1% solution in water)

Orange G - 0.5 ml (1% solution in water)

Glacial acetic acid - 2 ml

pH = 2.4-2.8

The fresh pollen grains were dusted into a drop of solution. The differentiation of aborted and viable pollen grains was based upon pollen color. Aborted pollen grains were green and viable pollen grains were red.

The fluorochrome reaction, FCR method (Heslop-Harrison and Heslop-Harrison, 1970), was used in an attempt to distinguish normal from abnormal pollen from partial-sterile plants. The procedure used was described by Gwyn and Stelly (1989), which is a modified fluorochrome reaction method. The modified method was prepared by mixing two stock solutions. Stock solution I was prepared by mixing 1.75 M sucrose, 3.23 mM boric acid (H_3BO_3), 3.05 mM calcium nitrate [$Ca(NO_3)$], 3.33

mM magnesium sulfate ($MgSO_4$), 1.98 mM potassium nitrate (KNO_3), and distilled water to complete volume. The stock solution II consisted of 7.21 mM fluorescein diacetate (Sigma Chemical Co., St. Louis, MO, Lot 53F-5022) dissolved in acetone. Working solutions were prepared daily adding 8 to 12 drops of stock solution II into 10 ml of stock solution I until the mixture became slightly milky. Fresh pollen grains were dusted into a drop of working solution and coverslip was applied. After 2 min the slides were observed under a Zeiss Standard Fluorescent Microscope equipped with epifluorescence, barrier filter 47 25 47, and excitation filter 47 72 18.

Pollen tube germination In an attempt to observe pollen tube growth down to the ovules to fertilize the egg cell, we designed an experiment to test for pollen tube in vitro germination. A factorial boric acid x sucrose combination was tested. The boric acid treatments were 0, 7.5, 15, and 30 ppm; sucrose treatments were 0, 5, and 10%. The best results were obtained with 7.5 ppm and 30 ppm of boric acid and 5% and 10% sucrose.

Freshly opened flowers were collected from growth chamber-grown plants early in the morning. Pollen grains from individual flowers were sprinkled onto the drop of boric acid x sucrose solution on each slide and allowed to grow at room temperature. After approximately one hour, the germinated

pollen grains, nongerminated pollen grains and burst pollen grains were counted. A pollen grain was considered germinated if the pollen tube had attained a length of at least 4 to 6 times the pollen grain diameter. Data are recorded as the percentage of pollen grains germinated or nongerminated in one observed microscope field.

Pollen morphology Scanning electron microscopy was used in an attempt to distinguish between normal and abnormal pollen grains from partial-sterile plants of PS-1 based on pollen morphology.

Freshly opened flowers were collected and pollen grains from individual plants were sprinkled on metallic tape glued to brass discs with silver cement. A 15 nm coat of gold-palladium (20:80) was applied using a Polaron E5100 sputter coater. Observations and photographs were made using a JEOL JSM-35 scanning electron microscope at accelerating voltages of 10-20 kV.

Female Gametophyte

Megagametogenesis For paraffin serial sections, flower buds of different sizes were collected and fixed in FAA solution for at least 24 hours. A gentle vacuum was used to enhance the penetration of fixative. The gynoecia were removed, both ends were cut with a razor blade, and the

gynoecia, and sometimes the ovule, were left in fixative over night. After fixation these buds were dehydrated through a graded ethanol/xylene series and infiltrated with Paraplast paraffin over a minimum period of 3 days. Sections were cut on a rotary microtome at 10 μm in either longitudinal or sagittal sections, stained with safranin, and overstained with fast green.

For resin sections, flower buds of different sizes were collected and fixed in a solution of 3% glutaraldehyde and 2% paraformaldehyde in sodium cacodylate buffer (0.1 M, pH 7.2) at room temperature. The gynoecia were dissected in the fixative, place under vacuum at 15 psi (6.89 kPa) for 1 hour, and then placed in fresh fixative at 4 C overnight. After fixation, washing was followed by three buffer rinses, postfixation in 1% osmium tetroxide (OsO_4) in same buffer for 4 hours at room temperature (22 C), and dehydration in a graded ethanol/acetone series. The specimens were embedded in Spurr's resin (Spurr, 1969), sectioned on a Reichert Ultracut E ultramicrotome at 1 to 2 μm thickness, and stained with toluidine blue.

Whole ovule clearing Ovules were dissected out of floral buds, fixed in FAA and stored for 24 hours at 4 C. After water wash, the ovules were stained in aqueous Mayer's Hemalum for 20-30 min and destained in 2% acetic acid for 10

minutes. Dehydration was carried out in an ethanol series to 100% ethanol. The latter was gradually replaced with methyl salicylate (Stelly et al., 1984). The ovules were mounted in methyl salicylate on slides and sealed with nail polish. Slides were stored at 4 C to prevent evaporation.

The specimens were observed in a confocal scanning laser microscope (CSLM). The Odyssey-CSLM used in this study incorporates a DIAPHOT-NIKON microscope. The specimen was illuminated by the focused light beam from an argon laser. The emission wavelength was 515 nm and excitation wavelength was 488 nm. A DIAPHOT-NIKON oil immersion objective 60/1.40 was used. The thickness of each recorded section was approximately 1μm, and the microscope was refocused at 2 μm between successive images. One ovule at a time was optically sectioned.

Early embryo abortion Partial-sterile plants from PS-1 were harvested and brought from the field into the laboratory in 1991, 1992 and 1993.

A pod-by-pod record of seed, aborted seed and aborted ovules was made for all tagged plants. Seed, seed abortions and ovule abortions within the pods were recorded as basal, middle, or apical in position. Percentage of ovule abortion was the number of ovule abortions against the total number of mature seeds, seed abortions, and ovule abortions.

RESULTS

Genetic Study

Linkage Tests The results of linkage test of PS-1 with Harosoy-w_4 are presented in Table 1. Segregation data, and χ^2 values for F_2 generation from crosses between PS-1 and Harosoy-w_4 were calculated for two consecutive years. In 1991 a total of 1212 F_2 plants and in 1992 a total of 1029 F_2 plants were classified. In both years the results fit the ratio of 9:3:3:1, that is the expected ratio for two independent loci. The percentage recombination was 55% ± 2 and 51% ± 2 respectively for both years. The results of $F_{2:3}$ segregation data (Table 2), confirm that we are dealing with two independent loci, each one segregating an 1:2:1 ratio (Table 2).

The results of linkage tests of PS-1 with CD-1 are shown in Table 3. Due to the weakness of F_2 yellow green plants, $F_{2:3}$ progenies tests were conducted. If the gene for plant color in CD-1 was linked to the gene for partial-sterility in PS-1, we would expect an excess of green and partial-sterile families as well as an excess of families segregating for plant color and fertile plants.

From a total of 219 $F_{2:3}$ families (Table 3), the observed plant color data fit the expected 1:2 ratio of nonsegregating to segregating. The seed set data fit the expected 1:2:1 ratio. These results suggest that the two loci are

independent, that is, the yellow-green locus is not linked to the locus that causes the partial-sterility in PS-1 soybean mutant.

The linkage test with Harosoy-w_4 and with CD-1 provided useful information that the gene in PS-1 is a single recessive gene. This explains the true breeding behavior of the PS-1 mutant after selfing or in segregating generations.

Allelism Tests The allelism tests data showed that the gene that causes partial-sterility in PS-1 mutant is not allelic to the gene(s) that cause partial-sterility in PS-2, PS-3, and PS-4 mutants.

If the gene in PS-1 was not allelic to the gene in PS-2, PS-3, and PS-4, we would expect that the F_2 generation to have families segregating in a ratio of three fertile plants to one partial-sterile plant, and plants within families segregating in a ratio of three fertile to five partial-sterile plants (Figs. 1-4).

In the F_2 population from crosses between PS-1 with PS-2, we observed two types of ratios, one segregated 3:1 and the other segregated 3:5. From 572 F_2 individual plants, the pooled χ^2 value fit the expected 3:1 ratio (Table 4). The homogeneity χ^2 value indicated that all progenies came from the same population (Table 4). In the F_2 population (Table 5) from crosses between PS-1 with PS-2, 786 individual plants also

were classified and the results gave a 3:5 ratio.

For the F_2 individual plants from crosses between PS-1 with PS-3 and PS-4 similar results were observed as with the F_2 crosses with PS-2. With PS-3 (Table 4), 294 plants were classified and the results fit the 3:1 ratio and gave a χ^2 value of 0.22. The F_2 individual plants (Table 5) from crosses with PS-1 and PS-3 gave 483 plants, the results fit the expected 3:5 ratio and gave a χ^2 value of 0.72. The homogeneity χ^2 value was 0.46 which suggested that the families were homogeneous.

The crosses with PS-4 also had F_2 families that segregated 3:1 (Table 4) and 3:5 (Table 5). The segregation data fit the expected ratios. The $F_{2:3}$ progeny tests confirmed the F_2 results. The gene in PS-1 is not allelic to the gene in PS-2, PS-3 and, PS-4. The F_2 families that segregated 3:1 were expected to segregate 1:1 or 1:1:2 in $F_{2:3}$ (Table 6) and those that segregated 3:5 were expected to segregate 3:5 or 1:2:5 in $F_{2:3}$ (Table 7). The observed data fit the expected ratios.

Cytological Study

Male Gametophyte

Microsporogenesis Microsporogenesis in partial-sterile 1 mutant was normal. During the sporogenous stage (Fig. 5), densely cytoplasmic sporogenous cells were surrounded directly by the tapetum. Microspore mother cells

(MMC) became isolated by callose shortly before meiosis (Fig. 6). After meiosis and cytokinesis the four microspores remain for a short time within the original callose layer as a tetrad (Fig. 7). Later the callose dissolves and the individual microspores are released. At this stage the microspores have numerous vacuoles, large plastids lacking starch, and a large nucleus appressed to the microspore wall. Mitosis and cytokinesis within the microspores produced the binucleate pollen grains, with a large vegetative cell and a small included generative cell.

The paraffin sections showed no differences between mature pollen grains from partial-sterile plants and pollen grains from fertile plants. All pollen grains were round, and stained very well with safranin-fast green (Fig. 8).

Pollen viability The pollen viability techniques that were used to evaluate the pollen grains of PS-1 mutant did not show any distinguishable differences between pollen grains from fertile plants and partial-sterile plants. With the simplest technique, I_2KI stainability, the pollen grains were all round, stained red-brown and were engorged with starch (Fig. 9). All pollen grains from PS-1 plants were normal as regards stainability and were identical to pollen grains from fertile plants. No differences were observed in the measurements of pollen grain diameter; the average diameter

was 30 μm.

Most nuclear dyes and vital stains used for assessing pollen viability have the disadvantage that they do not stain the aborted pollen grains (Alexander, 1969). The differential staining, that is a stain mixture of Malachite green, acid fuchsin, and orange G, can be used to differentially stain aborted and viable pollen grains of most angiosperms and the spores of gymnosperms (Alexander, 1969). The differential staining showed that the pollen grains from partial-sterile plants from PS-1 mutant were normal since they were stained red (Fig. 10).

According to Gwyn and Stelly (1989), most dyes and stains are reliable to reveal pollen quality but they do not evaluate the pollen ability to function and effect fertilization. The viability of the vegetative cell of the male gametophyte is correlated with the state of the plasmalemma; if this shows normal permeability, the cell is likely to be viable. Pollen grains with intact cell membranes are assumed to be viable, and can be distinguished readily by microscopic observation from those having damaged membranes. The sporopollenin of the exine of mature pollen grains often possesses a natural blue-green fluorescence (Heslop-Harrison and Heslop-Harrison, 1970). They developed the FCR method, which infers pollen viability by evaluating the integrity of the plasmalemma of the vegetative nucleus of the

microgametophyte.

In our study with the fluorochrome reaction, no difference was observed between pollen grains from PS-1 plants and pollen grains from normal plants. With this modified fluorochrome reaction, we observed that the background fluorescence was similar in all pollen grains (Fig. 11), meaning that the integrity of the plasmalemma was intact and the pollen grains were viable.

Pollen tube germination Pollen germination tests have been reported to provide reasonable estimates of pollen fertility. Fertilization is an event that is under control of a certain number of independent events. A simplified scheme for fertilization was presented by Dumas, Knox, and Gaude (1984). The pollen grains contact the stigma, the stigma recognizes the pollen, enzymes are released and the pollen grains are hydrated. Then the pollen grains start to germinate on the stigma and penetrate the pistil. Pollen tubes grow between the cells of the stylar-transmitting tissue and reach the micropyle and fuse with the egg cell.

During growth of the pollen tube toward the ovule in soybean, the generative cell divides and forms two male gametes, the sperm cells (Carlson and Lersten, 1987). Finally the pollen tube grows through the micropyle of the ovule, and enters the filiform apparatus of the degenerated synergids.

The pollen tube tip bursts and releases the two sperm cells. One sperm cell fuses with the egg cell and forms the diploid zygote, the other sperm cell fuses with the secondary nucleus forming the primary endosperm nucleus. This is called double fertilization.

We questioned whether the pollen tubes were able to grow down the style to fertilize the egg cell. If pollen tubes did not grow, the ovules aborted because of the failure to be fertilized. The best results were obtained at 10% sucrose combined with 30 ppm boric acid (Table 9). At this combination, the pollen germination was 87.8% (Fig. 12).

Pollen morphology No difference was observed in the morphology of pollen grains between partial-sterile plants and fertile plants. This indicates that they had the same size, shape and three colpi (Fig. 13).

Female Gametophyte

Megagametogenesis Megagametogenesis was observed using light microscopy and confocal laser microscopy. The soybean pistil is unicarpellate, and contains from one to four ovules. The ovules, which alternate along the placental suture, are bitegmic, campylotropous, and crassinucellate.

Ovules from PS-1 mutant were examined and they exhibited normal gametophytes. Flower buds at different stages were

sectioned and they showed normal development of the embryo sac. The archesporium was distinguished first as a group of cells (2-6) arising two or three layers beneath the epidermis of the developing ovule. One of these cells became the archesporium cell. At this stage the integuments began to appear as a small outgrowth at the base adjacent to the funiculus. The archesporial cell continued to enlarge into an elongate, oval-shaped cell and at this stage it is called megaspore mother cell (MMC) (Fig. 14). The MMC undergoes meiosis, resulting in a linear tetrad of haploid megaspores. The chalazal megaspore continues to grow while the three micropylar megaspores become disorganized and disintegrate. The chalazal megaspore undergoes three successive mitoses resulting in an eight-nucleate megagametophyte (Fig. 15).

The eight-nucleate gametophyte is called an embryo sac after the migration of one nucleus from each end toward the center, and subsequent wall formation. Egg cell (Fig. 16), polar nuclei (Fig. 17), two synergids (Fig. 18) and three chalazal antipodals (Fig. 19) are all contained within the large central cell.

The polar nuclei were the most variable component of the embryo sac in ovules from PS-1. Polar nuclei fusion was not always observed before fertilization, sometimes ovules at anthesis did not have the fused polar nuclei. This was observed not only in ovules from PS-1 but also in ovules from

normal plants. The nuclear envelope was not always observed in ovules from PS-1 but it was present in ovules from normal plants (Figs. 20-23).

The position of polar nuclei was not always close to the egg cell (Figs. 24 and 25). Ovules from partial-sterile plants had three classes of polar nuclei position in regards to the egg cell (Tables 10 and 11). The closest position was around 86.5 μm, the middle position was around 111.0 μm and the farthest position was around 171.1 μm. Young ovules from PS-1, 1 DBA and ovules at anthesis, with polar nuclei at third position are suspected to abort since all known aborted embryos had only the third position of polar nuclei. Ovules from normal plants had only the first two polar nuclei positions (Tables 10 and 11). Ovaries from normal plants five days after anthesis had embryos well developed. The orientation of polar nuclei also was not consistent in ovules from PS-1 mutant, sometimes instead of the center of the embryo sac it was on the side.

At time of fertilization, in the mature ovules the antipodals degenerated and disappeared. The central cell became engorged with starch and a filiform apparatus was observed in each synergid. Ovules from PS-1 mutant showed normal fertilization. The pollen tube grew down the style and the sperm fused with the egg. Ovules one day after anthesis showed evidence of fertilization, such as degenerated synergid

and sperm nucleus within the egg cell and near to the polar nuclei.

Some of the ovules from PS-1 mutant 2-3 days after anthesis, when observed with the light microscope, showed different stages of development, such as one ovule at two-celled embryo stage and the other ovule at fertilization stage. Ovules five days after anthesis, showed differences in size (Fig. 26) even under dissecting microscope. According to Peterson et al. (1992), ovules five days old are usually at the four-celled proembryo stage. In some ovaries from partial-sterile 1 mutant we observed embryos well developed and embryos already degenerating (Figs. 27-30).

Embryos at zygote stage were observed with intact polar nuclei. These results indicated that the double fertilization was not completed in these ovules as it was in other ovules that had zygote, proembryo and globular stages of embryo development.

Early embryo abortion Table 12 shows the percentage of early embryo abortion for partial-sterile 1 (PS-1) mutant in 1990, 1991, and 1993. The percentage of embryo abortion in two-ovule pods and three-ovule pods was similar.

DISCUSSION

Genetic Study

Linkage Tests Since PS-1 was obtained from a soybean population containing a suspected transposable element, and the transposable element had inserted at w_4 locus, we tested for linkage. F_2 segregation data, χ^2 values, and *P* values showed that the gene in partial-sterile 1 mutant was not linked to the w_4 locus (Table 1). The F_2 data fit the expected 9:3:3:1 ratio, since each locus segregated in a 3:1 ratio. The $F_{2:3}$ data supported the F_2 data that the two loci are inherited independently (Table 2).

Van Schaik and Brink (1958), based on their results in maize, suggested that transposable elements often transpose to linked sites. They worked with Modulator (**Mp**) which is an element associated with variegated pericarp and cob color in maize. This element is similar in action to the Activator (**Ac**) from maize. From the literature, we know that **Ac** has preferential transposition to closely linked sites (Greenblatt, 1984; McClintock, 1952); **Mu** elements in maize preferentially insert into nonmethylated sequences (Bennetzen, Brown, and Springer, 1988).

The PS-1 mutant and nine more mutants were recovered from a population containing a suspected transposable element and none of them were linked to the w_4 locus suggesting that if the transposable element is inserted at the w_4 locus, it moved 50

centiMorgans or more far from the w_4 locus. Since no molecular evidence is available to support the hypothesis that the w_4 locus has a transposable element insertion, our conclusions need to be confirmed molecularly.

The linkage test between PS-1 and CD-1 also suggested that the two loci are inherited independently. As the F_2 CD-1 yellow-green plants were too weak to classify for fertility, single- plant progeny rows of $F_{2:3}$ families were classified as either segregating for plant color or not segregating. For the PS-1 locus, the $F_{2:3}$ families were classified as segregating for fertility (normal and partial-sterile) and nonsegregating (normal or partial-sterile). The data indicated that PS-1 and CD-1 mutants segregate independently.

The results of linkage tests gave us information that the gene in PS-1 is a single gene recessive confirming the true breeding status of PS-1.

Allelism Tests The gene in PS-1 was nonallelic to the gene in PS-2, PS-3, and PS-4. Since they were not allelic, the expected ratio of fertile to partial-sterile plants in segregating F_2 generations, segregating 3:1 or segregating 3:5, was observed.

These results were expected due to the different genetic behavior of the partial-sterile mutants. The gene in PS-1 is a single recessive gene, so the partial-sterile plants do not

segregate at the next generation of selfing but are true breeding. In the next generation the gene in PS-2, PS-3, and PS-4 segregates fertile and partial-sterile plants in an approximate 1:1 ratio (Pereira and Palmer, unpublished results).

From previous studies, we observed that the gene in PS-2, PS-3, and PS-4 was not transmitted through the female parent (Pereira and Palmer, unpublished results). The PS-2, PS-3, and PS-4 genes behave as a lethal ovule, similar to the lethal ovule factors reported in maize by Singleton and Mangelsdorf (1940), Clark (1942) and Nelson and Clary (1952), so all ovules carrying the gene abort or have some abnormality that prevents fertilization of the ovule or endosperm development. Therefore, when the PS-2, PS-3, and PS-4 were used as female parents and PS-1 was the male parent, the partial-sterility trait was not transmitted to the next generation. The F_1 was fertile and the F_2 generation segregated 3:1 (fertile and partial-sterile) for PS-1. Thus the data presented for the allelism tests with PS-1 have PS-2, PS-3, and PS-4 only as the male parent.

When PS-2, PS-3, and PS-4 were used as male parent and PS-1 as female parent, two classes of F_1 plants were obtained: one fertile and the other partial-sterile. The F_1 fertile plants would segregate in a 3:1 ratio in the F_2, and the $F_{2:3}$ progenies would be segregating and nonsegregating. The $F_{2:3}$

nonsegregating families would be true breeding fertile or true breeding partial-sterile. The $F_{2:3}$ segregating families would segregate in a 3:1 ratio.

The partial-sterile F_1 plants would segregate in a 3:5 ratio (fertile : partial-sterile) in the F_2, and the $F_{2:3}$ progenies would be nonsegregating and segregating in a 3:5 ratio. In the 5 partial-sterile class, one class was double partial-sterile because it had both genes for partial-sterility, one from PS-1 and the other from PS-2 or PS-3 or PS-4. This double partial-sterile was easily identified in the field because it had green leaves and stem, delayed maturity and had a few two- and three-seeded pods.

Robinson-Beers, Pruitt, and Gasser (1992) working with complementation test with two sterile mutants in *Arabidopsis*, reported the occurrence of double mutants which exhibited a largely additive phenotype for sterility. In our study the double partial-sterile plants had an extreme reduction in two- and three-seeded pods. The ovule abortion data were 41% for two-seeded pods and 51% for three-seeded pods. This compares to the ovule abortion in PS-1 that was on average, 33% for two-seeded pods and 43.5% for three-seeded pods over three years. In this study the ovule abortion for normal plants was around 19.5 % and 19.9% for two- and three-seeded pods respectively over three years.

Robertson (1978) reported that only 20% of the **Mu**-induced

mutations in maize that appeared to be allelic were in fact allelic. Clark and Sheridan (1991) isolated 51 embryo-specific (**emb**) mutations from an active Robertson's Mutator maize stock and at least 88% represented independent mutation events.

The results of the allelism test were expected because in the PS-1 mutant, the partial-sterile plants do not segregate at the next generation of selfing, but are true breeding. In the next generation the PS-2, PS-3, and PS-4 mutants segregate fertile and partial-sterile plants in an approximate 1:1 ratio.

Robinson-Beers, Pruitt, and Gasser (1992) working with *Arabidopsis* reported two mutants **bel1** and **sin1**. Both mutants were chemically induced and have altered ovule development. Genetic analyses indicated that **bel1** segregates as a single recessive gene, and complementation tests showed that the two mutants are not allelic.

Cytological Study

Male Gametophyte Partial-sterile 1 mutant was recovered from a soybean population containing a transposable element. Since transposable elements move from one place to another in the genome and its movement can cause chromosome rearrangements such as deletions, inversions, and translocations, we thought that we might detect some degree of abnormality in the pollen grains.

Microsporogenesis Microsporogenesis was normal in the PS-1 mutant resulting in normal/viable pollen grains. Any disruption during the formation of pollen grains leads to some degree of sterility (Albertsen and Palmer, 1979). All mature pollen grains were round, intense red stained with safranin-fast green meaning normal/viable pollen grains.

Pollen viability The observations of pollen stained with iodine potassium iodide indicated no difference among pollen grains from partial-sterile plants; all pollen grains were plump and stained red-brown, presumably meaning fertile pollen.

With differential staining, we would be able to differentiate aborted pollen from viable pollen based on the color of pollen grains. All pollen grains that were analyzed from PS-1 had red pollen grains, meaning that the pollen grains were viable. Similar results were obtained with pollen from fertile plants. If the pollen grains were aborted they would be green, because they have only cell wall and the wall would be stained with Malachite green (Alexander, 1969).

With the modified fluorochrome reaction (FCR), all pollen grains from PS-1 had the same high intensity of fluorescence. Gwyn and Stelly (1989) using FCR were able to analyze 44 different true-breeding translocation homozygotes, respective F_1 heterozygotes, and three different

cytogenetically analyzed BC_1F_1 families. They found that translocation heterozygotes consistently produced higher frequencies of less intense fluorescent pollen grains than the homozygotes or normals. The method was efficient to distinguish between normal and abnormal pollen grains in cotton. In our study, we believe that the integrity of the plasmalemma of pollen grains from PS-1 is intact and that the vegetative nucleus of the male gametophyte was viable and that the pollen grains were able to effect fertilization. Based on these results we conclude that the pollen grains from PS-1 were normal, viable, and functional.

Pollen tube germination The pollen grains of PS-1 were germinated in sucrose/boric acid solutions and the results showed that pollen-tube germination was very good. The pollen germination indicated that the pollen grains from PS-1 were able to grow and fertilize. In serial sections of ovules one day after anthesis, we observed trace of pollen tubes entering into the micropyle.

Pollen morphology Scanning electron microscopy (SEM) was used in this study in an attempt to distinguish between normal and abnormal pollen grains from PS-1 plants. SEM observations showed that all pollen grains were normal; they had the same shape, size and three colpi.

After using all these techniques in an attempt to distinguish between normal pollen and abnormal pollen, we believe that the partial-sterility in PS-1 mutant is not due to pollen grain abnormalities. The pollen grains were normal with regards to microgametogenesis, viability, morphology, and germination. The pollen grains from the PS-1 mutant can effect fertilization.

Female Gametophyte The soybean pistil is unicarpellate, and contains from one to four ovules with three ovules the most common type. Polygonum type is the pattern of megagametophyte development.

According to Gottschalk and Kaul (1974) many genes affect the pathways for microsporogenesis and microgametogenesis, but male-fertile, female-sterile mutations are rare in the plant world. Cotton, sorghum, alfalfa, and common bean are some examples of crops in which female sterility has been reported (Stroman 1941; Casady, Heyne, and Weibel, 1960; Bingham and Hawkins-Pfeiffer, 1984). The sorghum mutant phenotype is due to complementary effect of two dominant genes; the other reported cases appear to be caused by single gene recessive mutations. Cytological observations of the alfalfa mutant revealed that incomplete integument development was associated with female sterility.

Megagametogenesis Megagametogenesis in ovules from PS-1 were examined and they exhibited normal development resulting in eight-nucleate megagametophyte (Kennell and Horner, 1985; Carlson and Lersten, 1987).

The fusion of the polar nuclei in normal plants was not consistent with the literature. Maheshwari (1950) and Carlson and Lersten (1987) report the fusion of polar nuclei before fertilization. Since we observed unfused polar nuclei in both genotypes, normal and partial-sterile, we believe that these discordant results are due to variability within the species.

We would like to mention the aspect of nuclear envelope that was not observed in some ovules from partial-sterile mutant and was observed in all ovules from normal plants. The nuclear envelope, which has a double membrane, under confocal microscope looks thick. Since all ovules were under the same clearing procedure and by using the CSLM, the sections are thin, and in perfect alignment, no artifacts are caused by embedding, cutting or staining (Fredrickson, 1990 and 1992). We do not believe that the absence of this structure was due to technique problems. Since we did not find any reference reporting problems in nuclear envelope, and we did not work with transmission electron microscope, we can not confirm that the absence of nuclear envelope also contributes to the abortion in PS-1.

The position and orientation of polar nuclei in ovules

from partial-sterile plants also was not consistent. According to literature (Maheshwari, 1950; Kennell and Horner, 1985; Carlson and Lersten, 1987) the fused polar nuclei are positioned against the top of the egg cell or directly above it. Our study showed that ovaries from partial-sterile 1 mutant had ovules positioned far from the egg cell. Ovaries five days after anthesis were observed with aborted embryos with intact polar nuclei/secondary endosperm nucleus far from the egg cell. We believe that these ovules were not fertilized and consequently endosperm formation did not occur. The position of polar nuclei/secondary endosperm nucleus prevents the fertilization in PS-1 mutant ovules.

Ovaries five days after anthesis in PS-1 mutant showed ovules with different sizes. Embryos five days old are reported to have, at least, four-celled proembryo, no starch grains in central cell and parietal free endosperm (Peterson et al., 1992). We observed that all small ovules were degenerating and the large ovules were fertilized. Serial and optical sections showed that the degeneration ranged from zygote stage to proembryo stage. Depending on the stage, the degenerated ovules were found with the polar nuclei intact, suggesting that the polar nuclei were not fertilized. The endosperm was not formed. In later stage of degeneration, such as late proembryo, we did not find even traces of either the free nuclear endosperm or endosperm itself, thus the embryo

died due to failure of endosperm formation caused by the failure of double fertilization. Similar results were reported by Cichan and Palser (1982) working with *Cichorium intybus*. They found that embryos from four-celled to the early globular stage were without endosperm and with polar nuclei at the central cell. They found that even though without endosperm the embryos were normal in appearance and developed up to about the 30-cell stage because they were supplied with nutrients by the periendothelial zone. At the globular stage the ovule normally is supplied with nutrients by the endosperm and the lack of endosperm formation caused the failure of embryo development (Cichan and Palser, 1982).

In soybean Chamberlin, Horner, and Palmer (1993) observed that the zygote lacked a cell wall, and was connected to the central cell by the plasmodesmata, suggesting that the zygote may absorb nutrients from the central cell. Folsom and Cass (1992) also reported that the plasmodesmata were responsible for the movement of nutrients that were formed by the breakdown of large amounts of starch, from the central cell to the proembryo. These findings could explain the nondegeneration of ovules up to the late proembryo stage because they might be supplied with metabolites by the central cell. At proembryo stages the embryo might be supplied with nutrients by the endosperm. If the endosperm is not formed, it would cause the failure of embryo development and eventually

early embryo abortion. It is assumed that the embryos aborted because, in general, endosperm is required for normal growth and differentiation of the embryo (Brink and Cooper, 1947). Embryo abortion is associated with unfertilized polar nuclei; non-migration of polar nuclei to the egg cell and consequently no fusion of sperm cell with polar nuclei; and the absence or failure of triple fusion even though the egg cell was fertilized (Mogensen, 1982; Vallania, Botta, and Me, 1987; Arthur, Ozias-Akins, and Hanna, 1993).

Early embryo abortion The percentage of ovule abortion was 33% and 43% for two-seeded and three-seeded pods respectively, for three years in partial-sterile plants from PS-1 mutant. In normal plants, the ovule abortion was 19.5% and 19.9% for two-seeded and three-seeded pods respectively over three years. Palmer and Heer (1984) recorded 40.6% of ovule abortion in heterozygous chromosome translocation plants and 15% ovule abortion and seed abortion in homozygous normal chromosome and homozygous translocated chromosome genotypes. They also observed that the ovule abortion in heterozygous chromosome translocation plants was equally frequent among all positions both in two- and three-ovule pods. Seed abortions were more frequent in the basal position of the pod than either the middle or apical positions in homozygous normal chromosome and homozygous translocated chromosome genotypes.

In our study the distribution of embryo abortions within the pod also was at random. Our results for ovule abortion and distribution of ovule/embryo abortion are in approximate agreement with their results.

According to Mogensen (1982) ovule abortion in angiosperms has been reported to be associated with a variety of factors such as lack of fertilization; failure of the embryo sac to complete its development; high temperatures, reduction of cell divisions in the nucellus, competition due to heavy crop load, suppression of other ovules in a given ovary by the first one to be fertilized, and blockage of the vascular trace leading to the ovule. We have evidence that the ovule abortion in PS-1 mutant is due to abnormal polar nuclei behavior. The polar nuclei do not migrate to their correct position. This condition would result in the lack of endosperm formation and, consequently early embryo abortion due to lack of nutrients.

Table 1. Number of F_2 plants from linkage test from crosses involving Harosoy-W4 with soybean partial-sterile 1 mutant (PS-1).

Phenotypes	Year		Total
	1991	1992	
Purple normal	694	576	1270
Purple partial-sterile	213	192	405
White normal	211	193	404
White partial-sterile	94	68	162
Total	1212	1029	2241
χ^2 (9:3:3:1)	6.49	0.11	4.41
P	0.09	0.75	0.22
%R	55.00	51.00	53.00
SE	±2.00	±2.00	±2.00
Purple:white	907:305	768:261	1675:566
χ^2 (3:1)	0.02	0.07	0.08
P	0.89	0.79	0.78
Normal:partial-sterile	905:307	769:260	1674:567
χ^2 (3:1)	0.07	0.04	0.11
P	0.79	0.84	0.74

Table 2. Number of $F_{2:3}$ progenies from crosses between Harosoy-w4 with soybean partial-sterile mutant 1 (PS-1).

	Flower color		
	Purple	Purple and white	White
Normal	17	46	22
Normal and partial-sterile	34	70	40
Partial-sterile	15	36	20
Purple:(purple and white):white		66:152:82	
χ^2 (1:2:1)		1.76	
P		0.41	
Normal:(normal and partial-sterile) : partial-sterile		85:144:71	
χ^2 (1:2:1)		1.80	
P		0.41	

Table 3. Number of $F_{2:3}$ progenies from crosses between soybean partial-sterile mutant 1 (PS-1) with CD-1.

	Plant color	
	Green	Green and yellow-green
Normal	14	43
Normal and partial-sterile	34	67
Partial-sterile	26	35
Green:(green and yellow-green)		74:145
χ^2 (1:2)		0.02
P		0.89
Normal:(normal and partial-sterile): partial-sterile		57:101:61
χ^2 (1:2:1)		1.31
P		0.60

Table 4. Number of plants of F_2 allelism test from crosses involving partial-sterile soybean mutants. The F_2 are expected to segregate 3:1 (normal:partial-sterile).

Cross	Fertility		$\chi^2_{(3:1)}$	P
	Normal	Partial-sterile		
PS-1 x PS-2				
Total	433	139	0.29	
Pooled χ^2 (1df)			0.15	0.70
Homogeneity χ^2 (2df)			0.14	0.93
PS-1 x PS-3				
Total	224	70	1.16	
Pooled χ^2 (1df)			0.22	0.64
Homogeneity χ^2 (2df)			0.94	0.62
PS-1 x PS-4				
Total	38	15	0.33	
Pooled χ^2 (1df)			0.31	0.60
Homogeneity χ^2 (1df)			0.02	0.89

Table 5. Number of F_2 families of allelism test from crosses involving partial-sterile soybean mutants. The F_2 are expected to segregate 3:5 (normal:partial-sterile).

Cross	Fertility		$\chi^2_{(3:5)}$	P
	Normal	Partial-sterile		
PS-1 x PS-2				
Total	297	489	0.00	
Pooled χ^2 (1df)			0.02	0.89
Homogeneity χ^2 (2df)			0.17	0.92
PS-1 x PS-3				
Total	172	311	1.18	
Pooled χ^2 (1df)			0.72	0.40
Homogeneity χ^2 (2df)			0.46	0.80
PS-1 x PS-4				
Total	41	79	0.96	
Pooled χ^2 (1df)			0.57	0.45
Homogeneity χ^2 (2df)			0.39	0.82

Table 6. Number of $F_{2:3}$ families from allelism test between soybean partial-sterile mutants segregating 3:1 in the F_2. Families are expected to segregate 1:1 or 1:1:2 (nonsegregating normal:nonsegregating partial-sterile:segregating).

Cross	Number of entries			$\chi^2_{(1:1)}$	P	$\chi^2_{(1:1:2)}$	P
	nonsegregating		segregating				
	N[a]	PS[b]	N and PS				
PS-1 x PS-2							
Total	7	7	17	0.00	1.00	0.29	0.85
PS-1 x PS-3							
Total	15	12	33	0.66			
Pooled χ^2 (1df)				0.60	0.44	0.90	0.55
Homogeneity χ^2 (3df)				0.06	0.99		
PS-1 x PS-4							
Total	11	15	27	0.03			
Pooled χ^2 (1df)				0.02	0.89	0.62	0.75
Homogeneity χ^2 (1df)				0.01	0.92		

a: Normal
b: Partial-sterile

Table 7. Number of $F_{2:3}$ families from allelism test between soybean partial-sterile mutants segregating 3:5 in the F_2. Families are expected to segregate 3:5 or 1:2:5 (nonsegregating normal:nonsegregating partial-sterile:segregating).

Cross	Number of entries			$\chi^2_{(3:5)}$	P	$\chi^2_{(1:2:5)}$	P
	Nonsegregating		Segregating				
	N[a]	PS[b]	N and PS				
PS-1 x PS-2							
Total	6	10	25	0.11	0.76	0.14	0.93
PS-1 x PS-3							
Total	14	25	81	1.86			
Pooled χ^2 (1df)				1.28	0.26	1.38	0.50
Homogeneity χ^2 (3df)				0.58	0.90		
PS-1 x PS-4							
Total	13	24	55	1.22			
Pooled χ^2 (1df)				0.29	0.59	0.35	0.85
Homogeneity χ^2 (2df)				0.93	0.63		

a: Normal
b: Partial-sterile

Table 8. Percentage pollen germination from PS-1 mutant in sucrose x boric acid solutions.

Sucrose (%)	Boric acid (ppm)	Germination (%)
0	0.0	0.0
5	0.0	0.0
10	0.0	24.5
0	7.5	74.0
5	7.5	69.0
10	7.5	60.0
0	15.0	47.5
5	15.0	47.5
10	15.0	70.5
0	30.0	37.5
5	30.0	45.0
10	30.0	87.5

Table 9. Percentage pollen germination from normal plants and PS-1 mutant plants in 10% sucrose and 30 ppm boric acid solution.

Plant	Normal	PS-1
1	90.0	93.0
2	89.5	84.5
3	92.0	86.0
4	88.5	83.5
5	85.0	92.0
Average	89.0	87.8

Table 10. Number of ovules observed in PS-1 and normal plants in regard to polar nuclei position.

	Polar nuclei position (μm)		
	<100	100-140	>140
PS-1			
Number of ovules	19	13	38
Average	86.5	111.0	171.7
Range	65.5 to 99.2	100.6 to 133.6	151.2 to 188
Normal			
Number of ovules	36	34	
Average	87.6	120.1	
Range	83.2 to 97.6	102.8 to 140	
χ^2		52.64**	

** significant difference at the 0.01 level

Table 11. Number of ovules observed in PS-1 mutant and normal plants in regard to polar nuclei position and ovary stage.

Polar nuclei position (μm)	Number of ovules		
	PS-1		
	1 DBA	Anthesis	5 DAA[1]
< 100	10	9	0
100-140	9	4	0
> 140	5	7	26
	Number of ovules		
	Normal		
	1 DBA	Anthesis	
< 100	16	20	
100-140	20	14	
> 140	0	0	

DBA: Day before anthesis.

DAA: Day after anthesis.

1/ Ovaries with aborted embryos.

Table 12. Percentage of embryo abortion in partial-sterile mutant 1 (PS-1) for three years, Ames, Iowa.

	Year	Number of plants	Percentage of embryo abortion in pods with	
			2 seeds	3 seeds
PS-1	1990	30	34.1	44.2
Normal		14	17.8	25.1
PS-1	1991	30	34.9	41.5
Normal		32	21.3	17.2
PS-1	1993	5	31.0	45.0
Normal		n.d.	n.d.	n.d.

n.d.: no data

Cross between PS-1 and PS-2: F_1 seeds

♀ Gamets	♂ Gametes	
	AB	**Ab**
aB	**AaBB** (Normal/Fertile)	**AaBb** (Partial-sterile)

Fig. 1: Scheme showing how we obtained the ratio 3:5 in allelism test between PS-1 as female parent with PS-2, or PS-3, or PS-4 as male parent.

Assumptions:

1) **a**: Single recessive gene for partial sterility in PS-1 mutant
2) **b**: Single recessive gene for partial sterility in PS-2 (or PS-3, or PS-4) mutant, transmitted only through the male parent
3) PS-1 and PS-2 (or PS-3 or PS-4) are nonallelic
4) Genotypes

   PS-1: aaBB

   PS-2 or PS-3, or PS-4: AABb

Selfing of F_1 plants (AaBB): F_2 generation

AaBB (N)

♀ Gametes	♂ Gametes	
	AB	**aB**
AB	AABB (Normal)	AaBB (Normal)
aB	AaBB (Normal)	aaBB (Partial-sterile)

3 (N) : 1 (PS-1)

Fig. 1. (continued)

Selfing of F_1 plants (AaBb): F_2 generation

AaBb (PS-2)

♀ Gametes	♂ Gametes			
	AB	**Ab**	**aB**	**ab**
AB	AABB (Normal)	AABb (PS-2)	AaBB (Normal)	AaBb (PS-2)
Ab[1]	ABORTED	ABORTED	ABORTED	ABORTED
aB	AaBB (Normal)	AaBb (PS-2)	aaBB (PS-1)	aaBb (DPS[2])
ab[1]	ABORTED	ABORTED	ABORTED	ABORTED

3 (N) : 5 (PB)

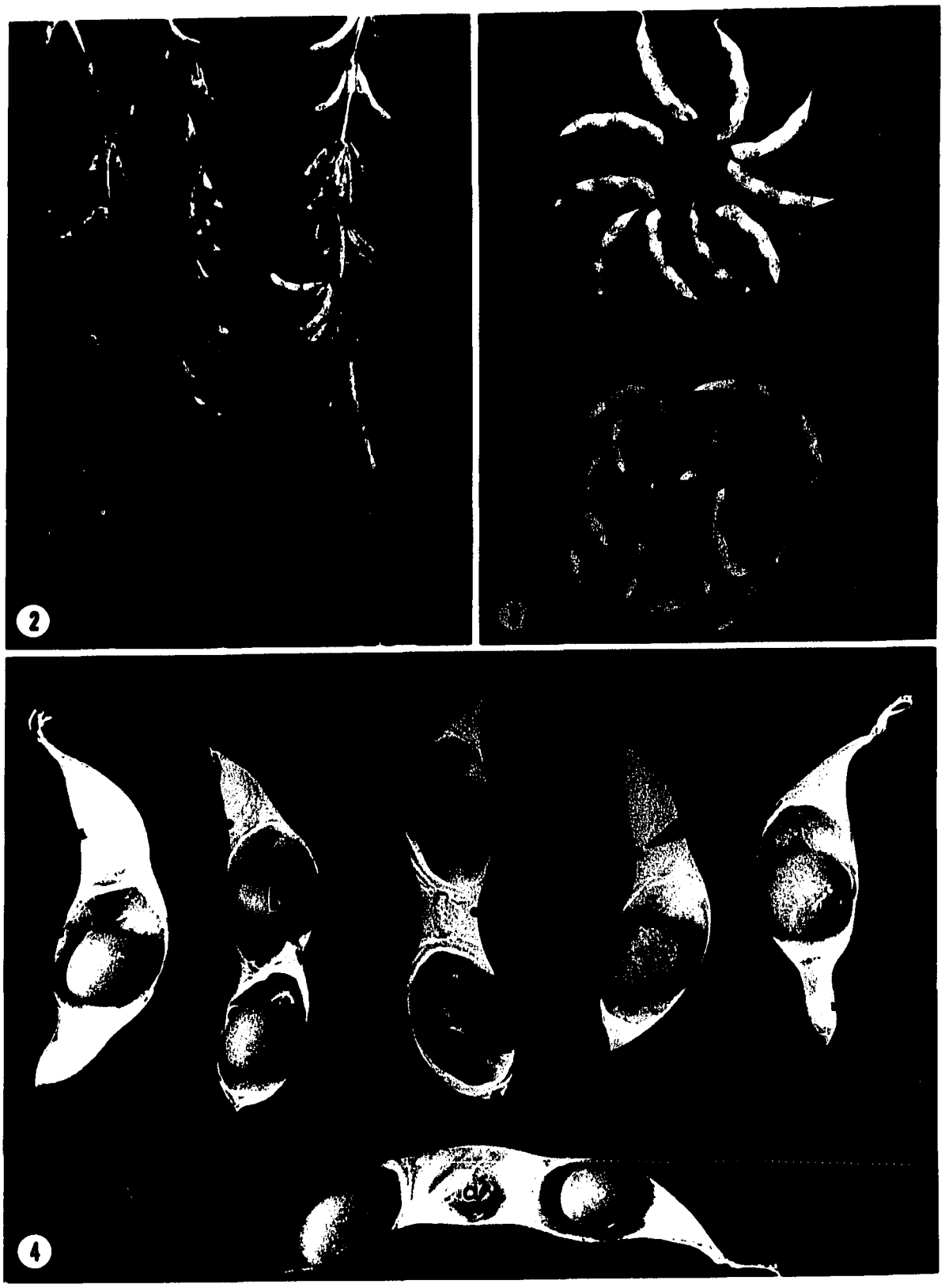
1/ Ovules carrying the **b** allele from PS-2 or PS-3 or PS-4 are aborted

2/ DPS: Double partial-sterile

Fig. 1. (continued)

Figs. 2-4. Comparison between PS-1 plants and normal soybean plants.

2. Seed set comparison between double partial-sterile (left), partial-sterile (center), and normal soybean plants (right).
3. Pod comparison between normal (top) and partial-sterile plants (bottom).
4. Soybean pod showing a seed abortion (a), early embryo abortion (arrow), and normal seed.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Figs. 5-8. Microsporogenesis in PS-1 mutant.

5. Sporogenous mass stage. MMC nuclei are small (arrow). Anther wall layers have not differentiated. Bar = 10μm
6. Meiotic stage. MMC nuclei are enlarged, and the tapetum is becoming differentiated. Bar = 25μm
7. Coenocytic tetrads of microspore nuclei. Microspore tetrads are embedded in callose in a tetrahedral arrangement. Bar = 20μm
8. Anther with mature pollen grains. tapetal cells already disintegrated.Bar = 25μm

⑤

④

⑦

⑥

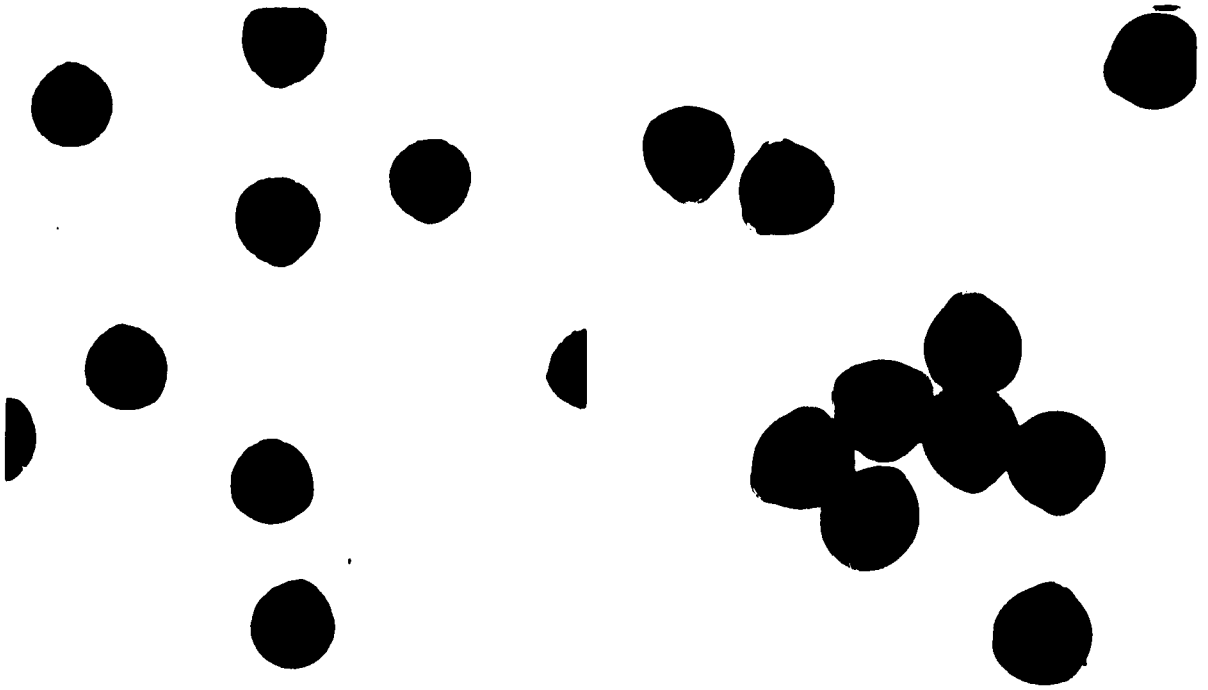
Figs. 9-12. Pollen grains from PS-1 mutant stained with different techniques.

9. Pollen grains stained with I_2KI, showing pollen grains engorged with starch. Bar = 10μm

10. Pollen grains stained with Malachite green, fuchsin acid, and orange G (differential staining), showing viable pollen grains. Bar = 10μm

11. Pollen grains stained with fluorescein diacetate, FCR method. All pollen grains had bright fluorescent background. Bar = 20μm

12. Pollen-tube germination in sucrose x boric acid solution. Bar = 25μm



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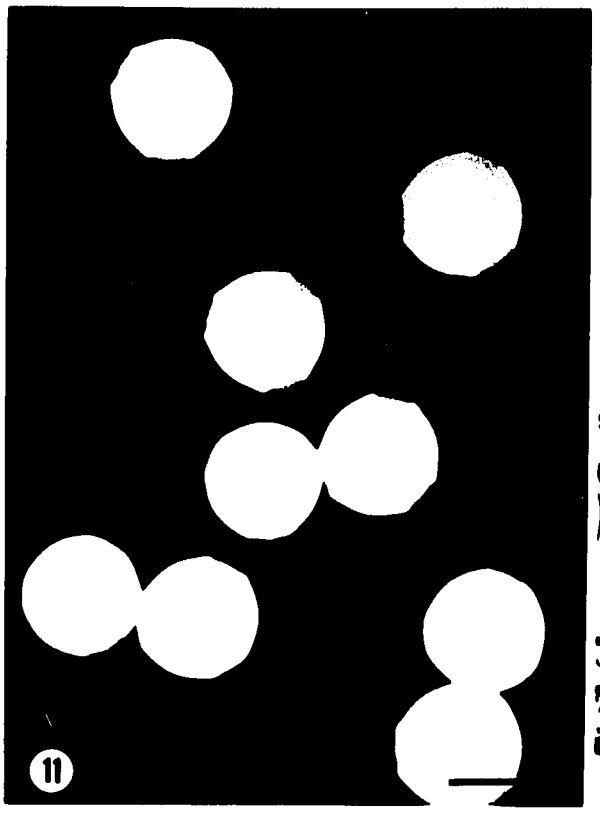

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(10)

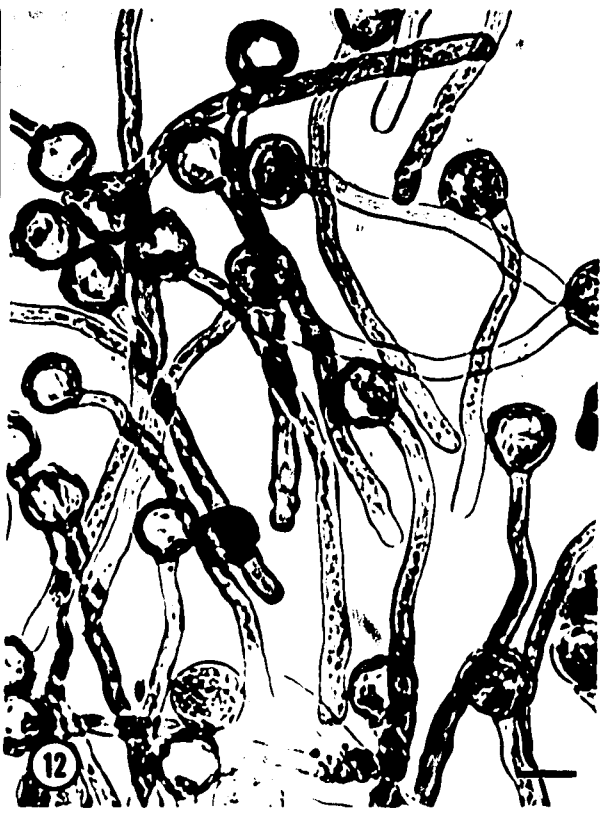

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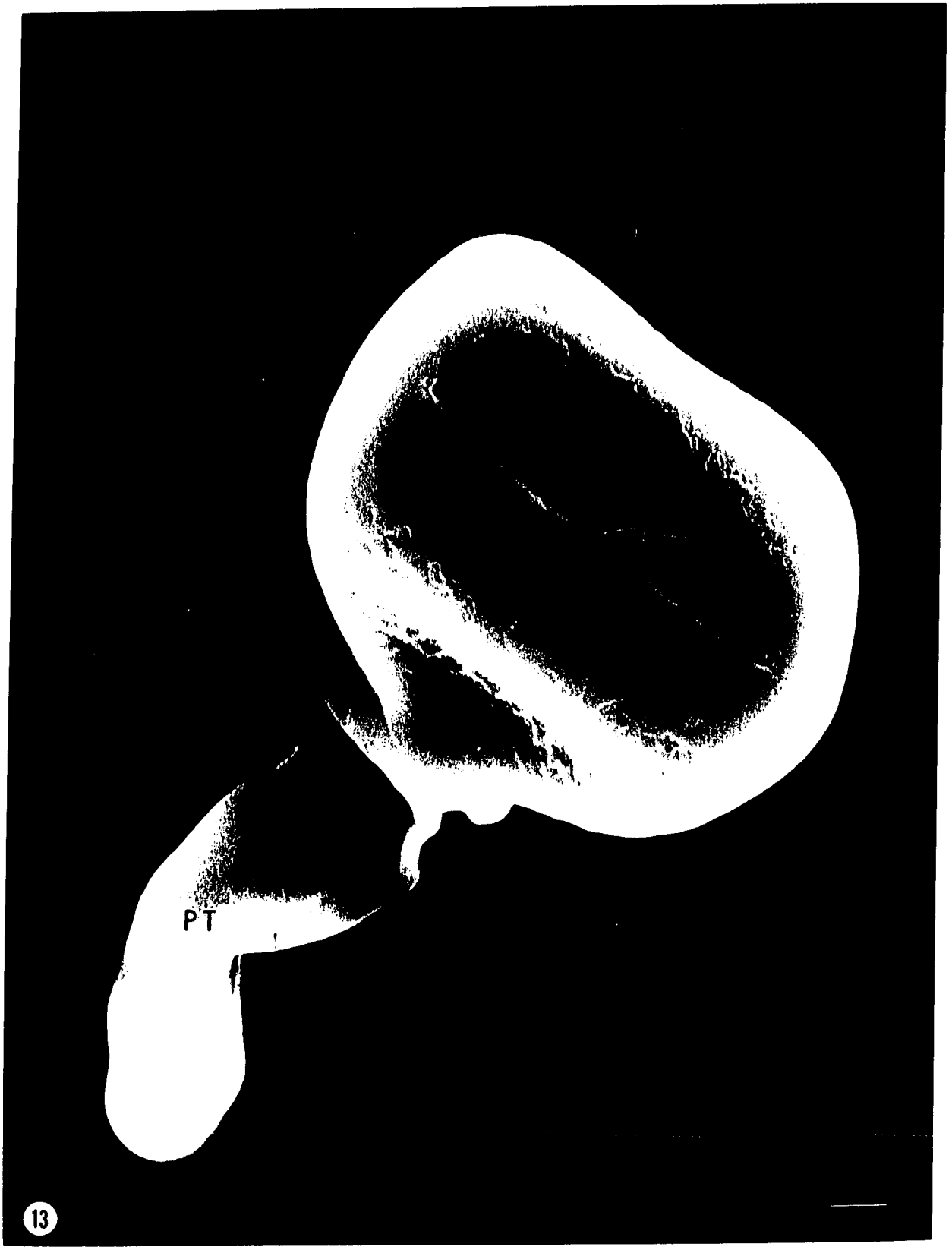
Fig. 13. SEM micrograph showing pollen grain from PS-1 mutant with colpi (arrow) and pollen tube (PT). Bar = 5μm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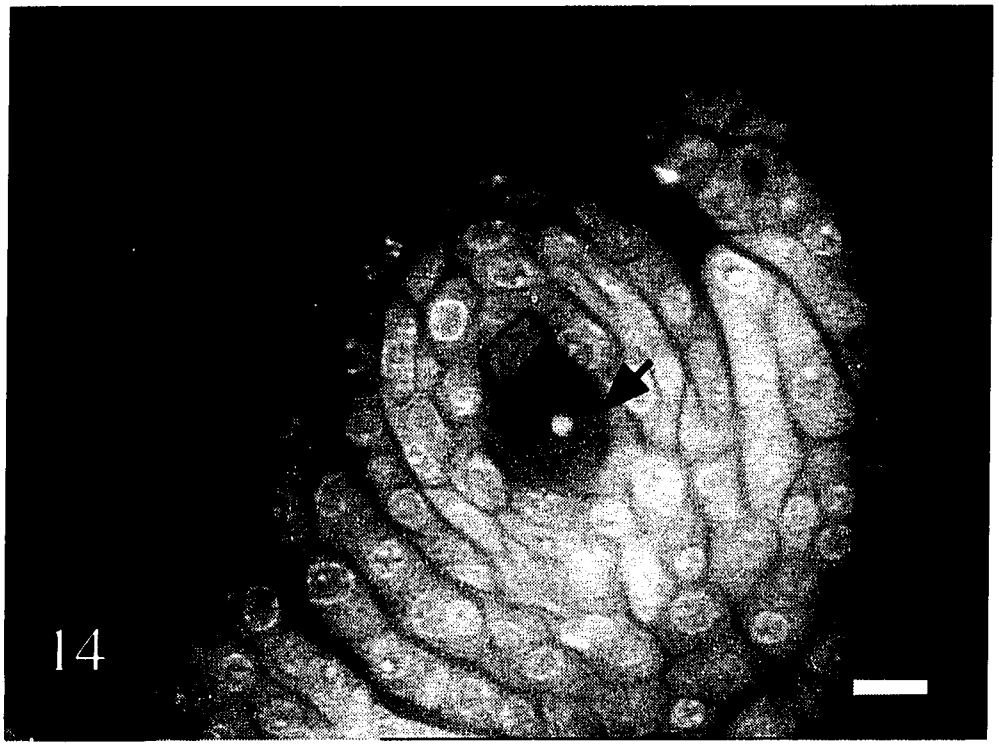
Figs. 14-15. Megagametogenesis in ovules from PS-1 soybean mutant.

14. Functional megaspore showing the nuclei (arrow) and the integuments growing. Bar = 10μm

15. Mature gametophyte of partial-sterile plant showing the egg cell (black arrow) at micropylar end and secondary endosperm nucleus (arrow) showing the nucleolus. Bar = 10μm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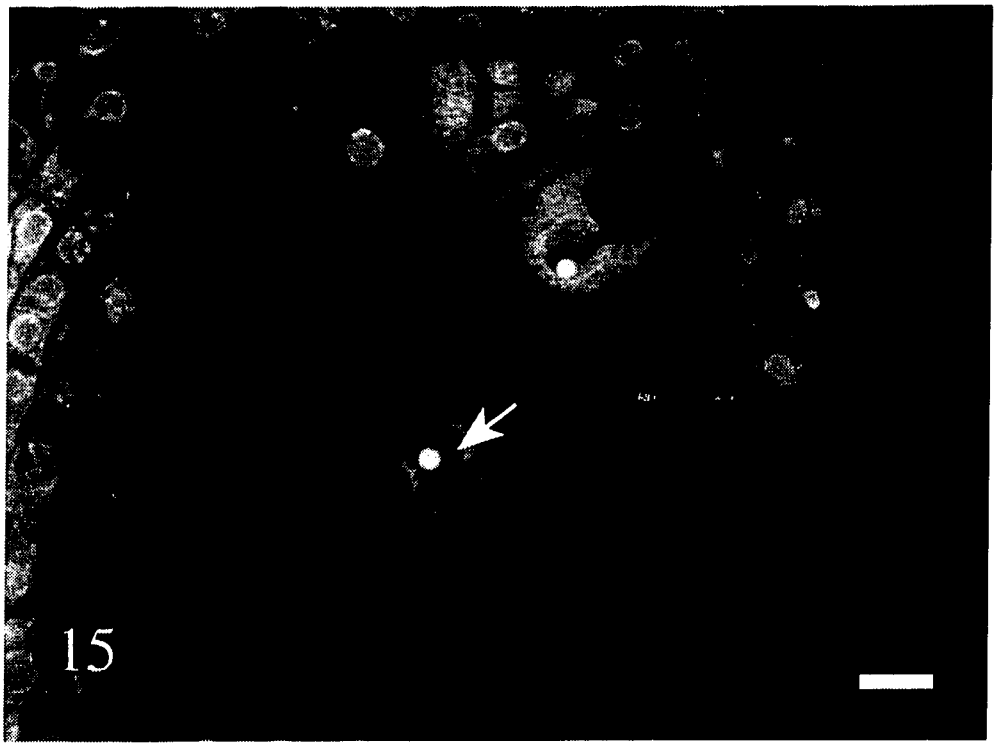
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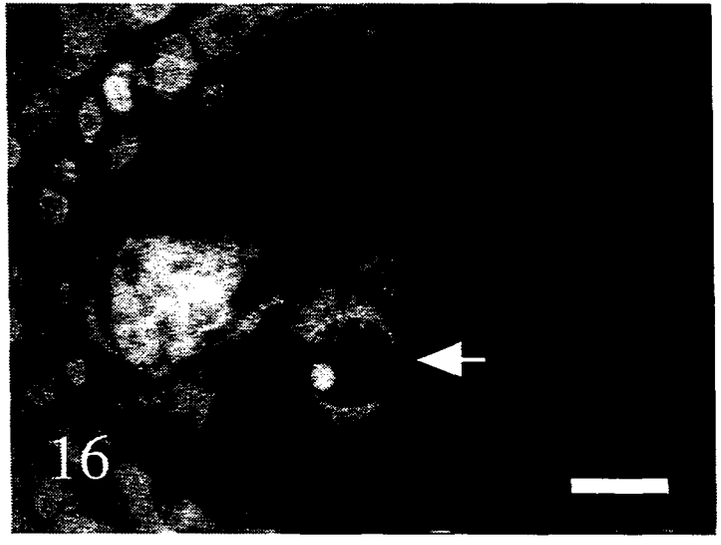
Figs. 16-19. Megagametophyte in ovules from PS-1 soybean mutant.

16. Egg cell at micropylar end showing the big vacuole and the cytoplasm with nuclei (arrow). Bar = 10μm
17. Central cell showing the unfused polar nuclei with nuclear envelope (arrow). Bar = 10μm
18. Synergids at micropylar end (arrow). Bar = 10μm
19. Antipodals at chalazal end (arrow). Bar = 10μm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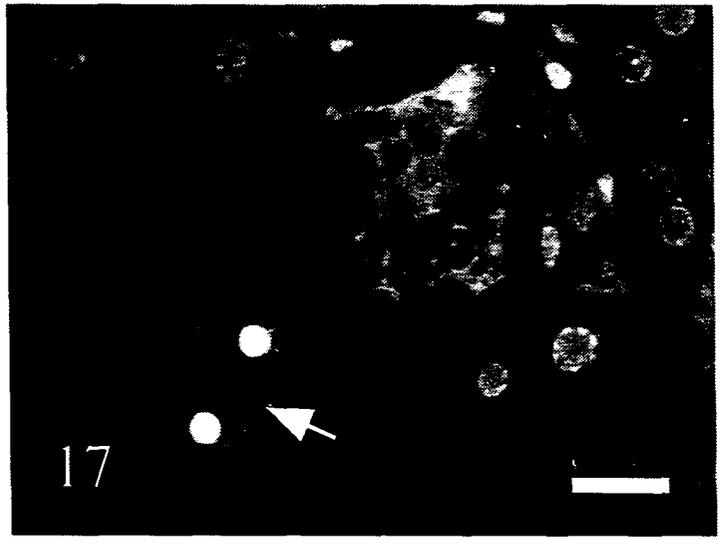
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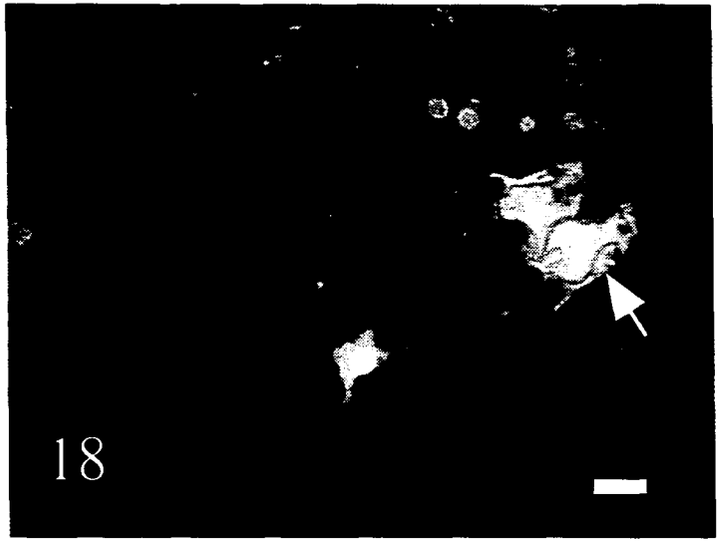
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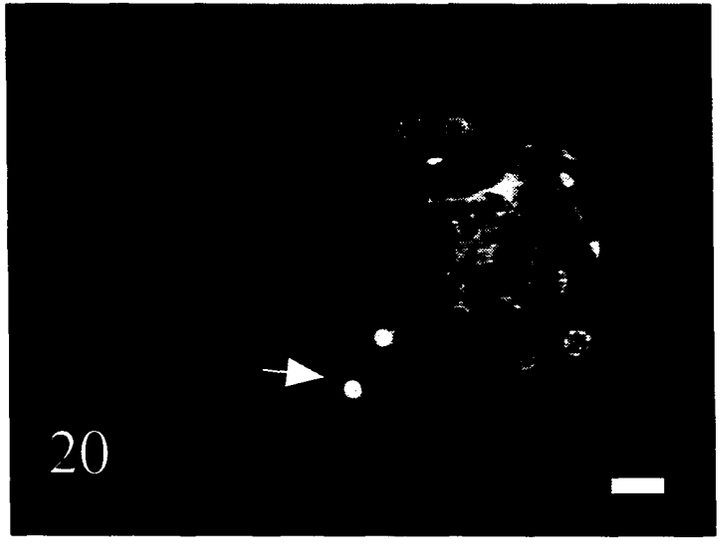
Figs. 20-21. Polar nuclei (Fig. 20) and secondary endosperm nucleus (Fig. 21) with nuclear envelope (arrow) in ovules from PS-1 mutant. Bar = 10μm

Figs. 22-23. Polar nuclei (Fig. 22) and secondary endosperm nucleus (Fig. 23) without nuclear envelope (arrow) in ovules from PS-1 mutant. Note the egg cell (arrowhead) at micropylar end (Fig. 23). Bar = 10μm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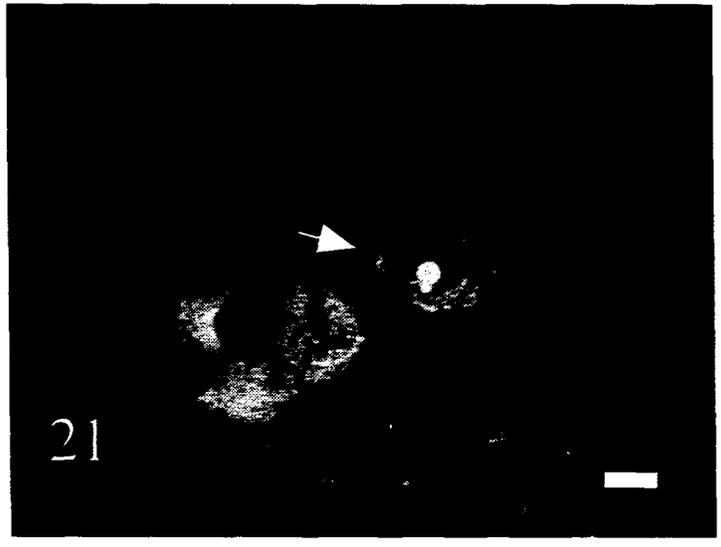
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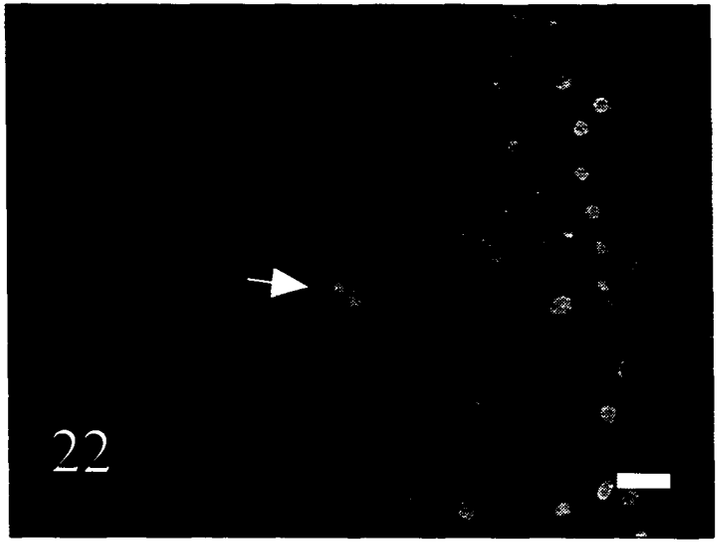
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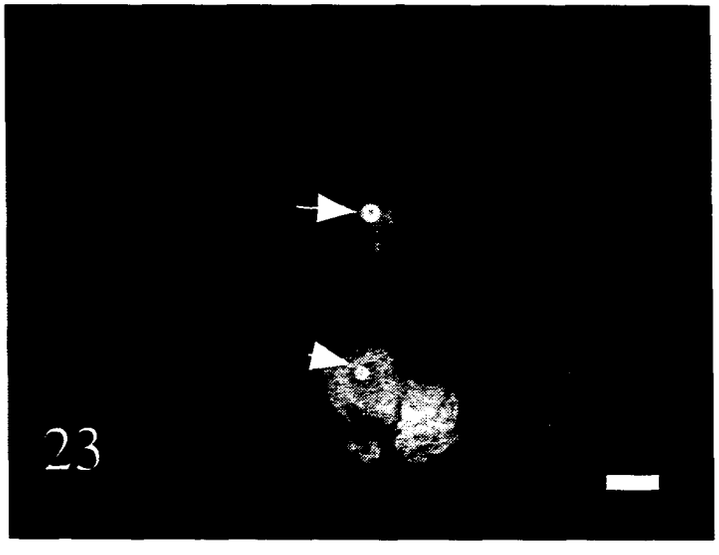
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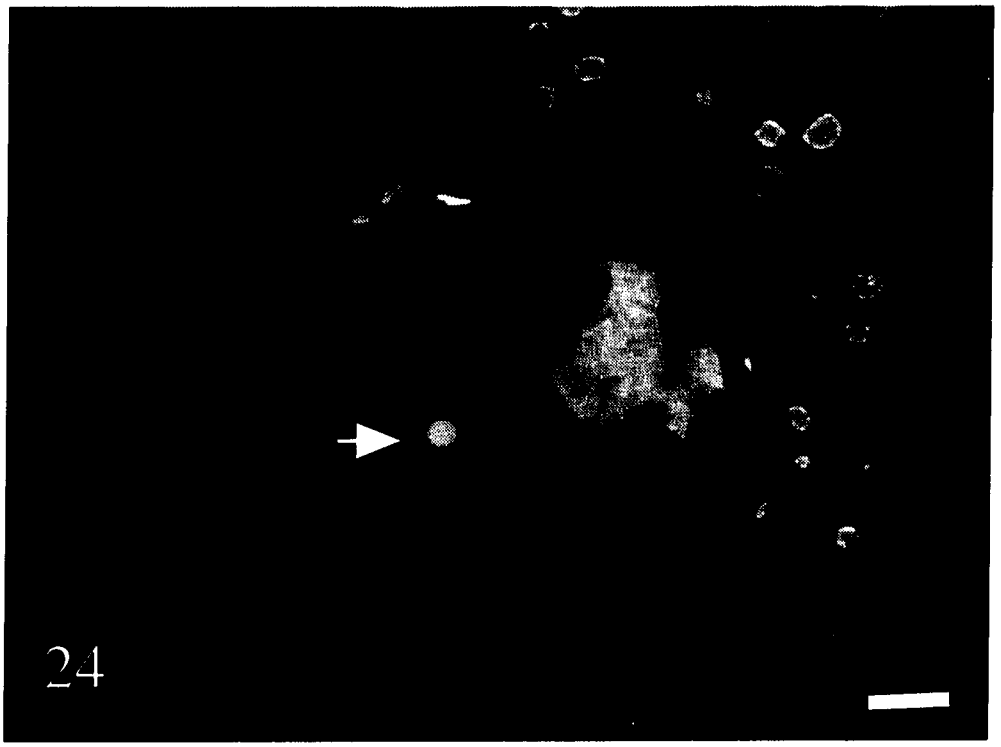
Fig. 24. Secondary endosperm nucleus (arrow) positioned close to the egg apparatus. Bar = 10μm

Fig. 25. Secondary endosperm nucleus (arrow) positioned far from the the egg apparatus. Bar = 10μm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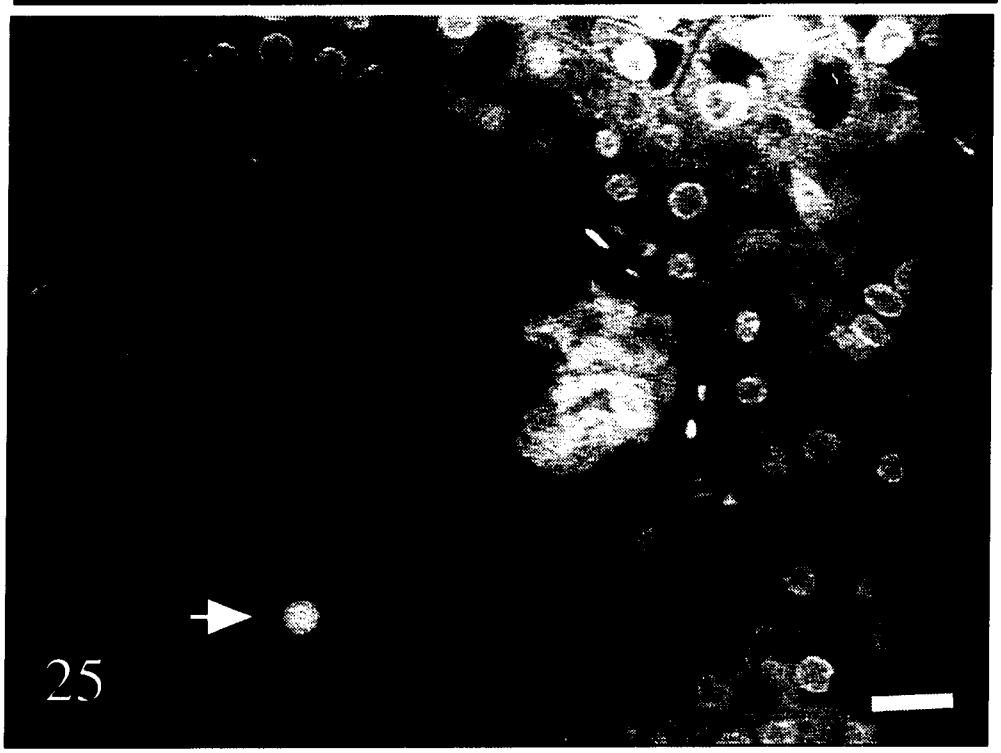
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Fig. 26. Soybean ovary five days after anthesis showing two fertilized ovules and one with embryo abortion (arrow). Bar = 100μm

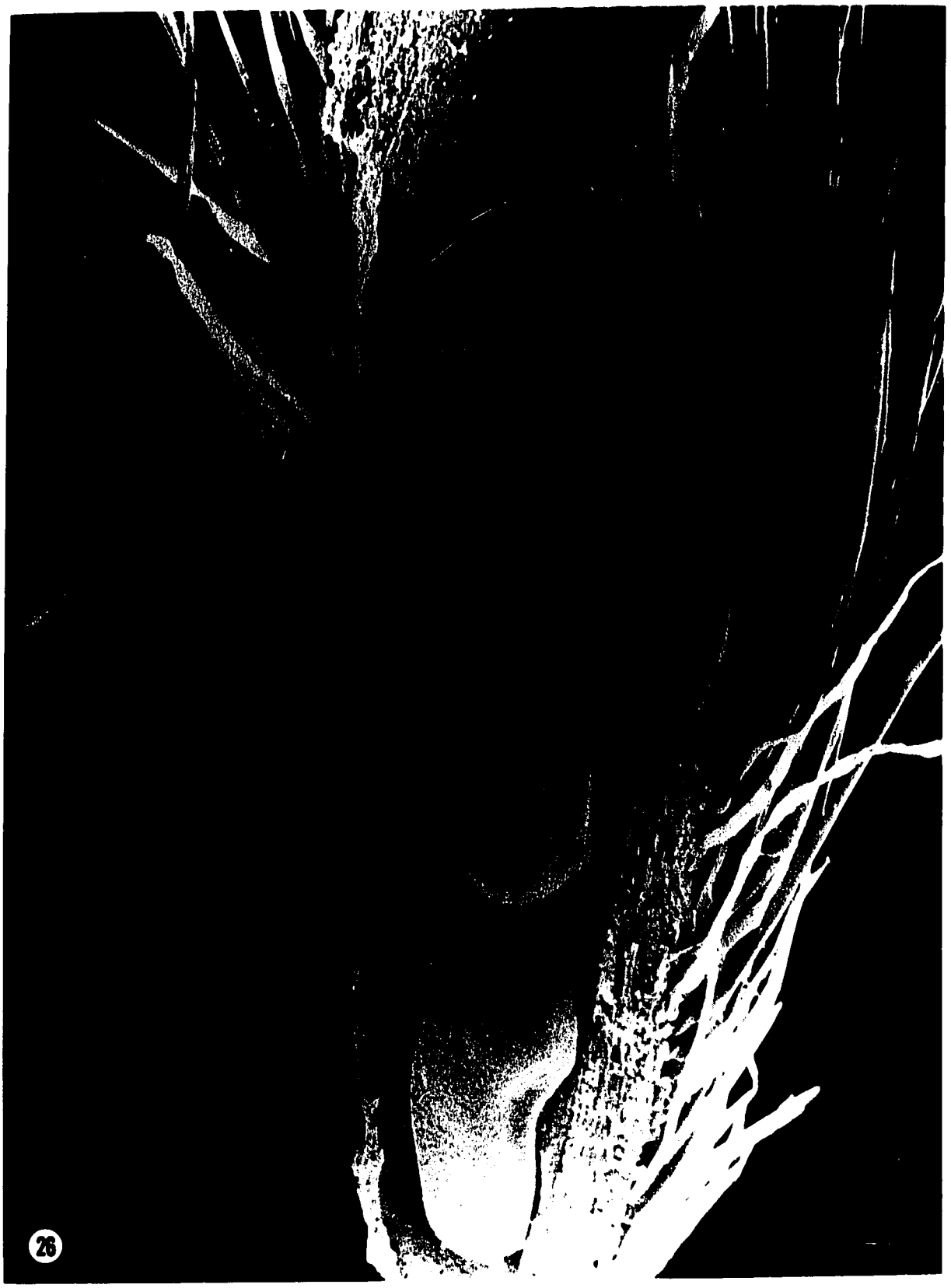
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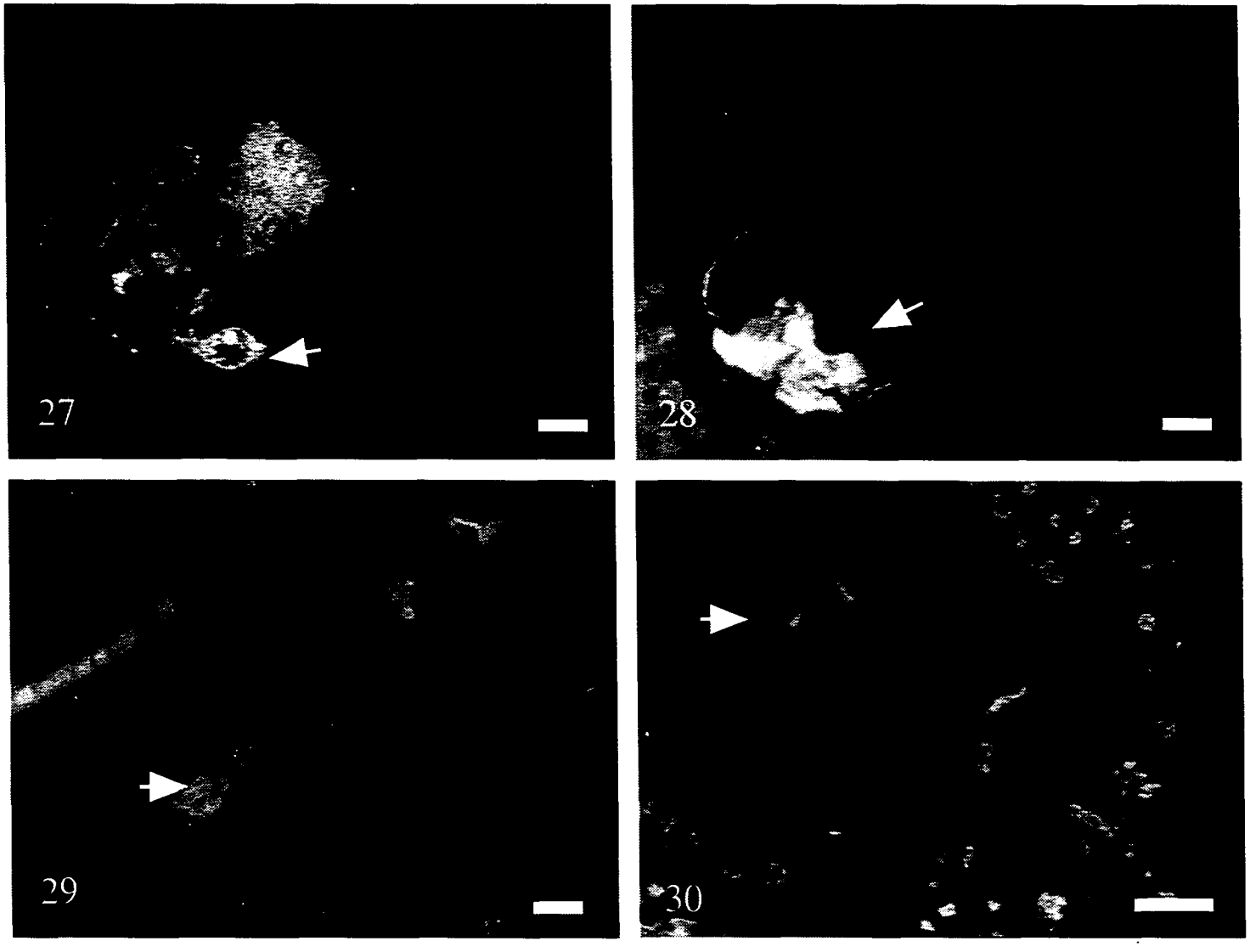
Fig. 27. Embryo at proembryo stage in ovaries five days after anthesis from PS-1 mutant. Note the free nuclear endosperm (arrow). Bar = 10μm

Figs. 28-30. Aborted embryo showing traces of degenerated young proembryo (arrow). Bar = 10μm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GENETIC AND CYTOLOGICAL ANALYSES OF THREE PARTIAL-STERILE (PS-2, PS-3, AND PS-4) MUTANTS IN SOYBEAN (*Glycine max*; LEGUMINOSAE)

A paper to be submitted to the Genome

Telma Nair S. Pereira and Reid G. Palmer

ABSTRACT

Soybean partial-sterile mutants 2, 3, and 4 (PS-2, PS-3, and PS-4) were recovered from a transposon tagging study. The objectives were to study the inheritance, linkage, allelism, and reproductive biology of the three mutants. For inheritance and linkage tests, PS's mutants were crossed to Harosoy-w_4, and to chlorophyll-deficient mutant, CD-1 and CD-5, also recovered from the tagging study. For allelism tests reciprocal crosses were made among the three partial-sterile mutants (PS-2, PS-3, and PS-4). Inheritance studies indicated that PS-2, PS-3, and PS-4 are maintained as heterozygote and upon self-pollination segregate 1 fertile : 1 partial-sterile. Linkage results indicated that the gene for partial sterility in the PS-2, PS-3, and PS-4 was not linked either to w_4 locus or to the genes for chlorophyll-deficiency. Linkage tests and allelism tests indicated that the gene in partial-sterile

mutants was not transmitted through the female when partial-sterile mutants were used as female parents. Results of pollen staining indicated no difference in morphology, stainability or fluorescence between normal and partial-sterile genotypes from partial-sterile mutants. Megagametogenesis indicated that the ovule abortion in PS's mutants was due to failure of fertilization. The partial-sterile plants ovules had normal embryo sac development but the failure of double fertilization caused the ovule abortion.

Key words: partial-sterile, transposable element, ovule abortion

INTRODUCTION

Transposable elements are segments of DNA that possess the ability to move to new locations in the genome. The movement of a transposable element can generate mutations or chromosomal rearrangements that can affect the expression of other genes. Deletions, duplications, translocations and inversions are some of many chromosomal rearrangements that can be produced by chromosome breakage (Saedler and Nevers, 1985; Nevers et al. 1986).

Since Barbara McClintock (1948) reported the occurrence of a transposable element in maize, transposable elements have

been found in at least 35 mono- and dicotyledonous plant species (Nevers et al. 1986). In soybean, three mutable alleles (Y_{18}-m, W_4-m, and r-m) and several insertion elements (Tgm family) have been reported (Peterson and Weber 1969; Groose et al. 1988; Chandlee et al. 1989; Goldberg et al. 1983). The coding sequence and 5′-flanking region of the Gy4 glycinin gene has a transposable element-like sequence in the hypervariable region (HVR)(Xue et al. 1992). However, there is no molecular evidence for transposition in either these mutable alleles or insertion elements in soybean.

The allele W_4-m is recessive to wild type and represents a mutation at the W_4 locus. The population containing the mutable allele is called W_4-mutable line and the mutable plants have both near white and purple flowers, as well as flowers of mutable phenotype with purple sectors on near-white petals (Groose et al. 1990; Palmer et al. 1989).

Genetic evidences suggest that the W_4-m is controlled by a transposable element Groose et al. (1990). The W_4-m reverts from an unstable form to a stable dominant form at a rate of 6.2% of reversion per allele per generation and the change to a wild type allele is heritable (Palmer et al. 1989); The allele can revert at different times during development. The size of revertant sectors on mutable plants is dependent on the developmental timing of reversion of W_4-m. Variability in development timing of reversion also is observed in flowers of

mutable plants (Groose et al., 1988). The loss of mutability can be due to imprecise excision of an element from the w_4-m locus, leaving behind a permanently impaired allele. The w_4-m allele has generated a number of pale derivatives, that also can be due to an imprecise excision of the element or the insertion of the element at new locations within the locus. The recovery of new mutants at other loci among the progeny of germinal revertants also suggested that w_4-mutable line had a transposable element inserted at the w_4 locus. All these genetic evidences support the hypotheses that w_4-m is controlled by a transposable element.

Several new mutants were isolated in a transposon tagging study in the w_4 mutable line, such as mutants for chlorophyll-deficiency (CD-1 to CD-8), mutants for root necrosis (NR-1 to NR-3), mutants for partial sterility (PS-1 to PS-4), and a mutant for near sterility (Palmer et al. 1989).

Partial-sterile 2, 3, and 4 (PS-2, PS-3, and PS-4) soybean mutants were recovered from an F_{11} family which was descended from a single F_9 plant of the Asgrow Mutable line. The F_{11} families were segregating for normal and partial-sterile plants (Groose and Palmer 1987). The normal plants in these families produced two-, three- and four-seeded pods. The partial-sterile plants in PS's mutants are characterized by reduced number of seeds per pod and plants are easy to identify at maturity by the high number of one- and two-

seeded pods. The reduction in number of seeds per pod can be the result of ovule abortion or very early embryo abortion.

In soybean sterility-inducing mutations are of two types:i) male-sterile, female-fertile (MS-FF); ii) male-sterile, female-sterile (MS-FS). The former, (MS-FF), mutations selective eliminate male reproductive function, but do not greatly affect the female reproductive function. The later, (MS-FS), mutations affect both male and female reproduction (Graybosch and Palmer 1988). In soybean, asynaptic sterile mutants, **st2** and **st3**, and desynaptic sterile mutants, **st4** and **st5**, are highly male and female sterile. The asynaptic mutant genes cause the failure of homologous chromosomes to pair during the first meiotic prophase and desynaptic mutant genes control chiasma formation or prevent chiasma formation (Gottschalk and Kaul 1974). The major consequence of desynaptic mutant genes is the production of gametes with various degrees of chromosomal imbalance, due to unequal chromosome distributions at anaphase I and II. This gametic imbalance leads to almost complete sterility in both meiocytes (Palmer 1974).

In the soybean flower-structure mutant, stamen filaments failed to elongate normally and the ovules were positioned abnormally, either side by side or tightly appressed to the ovary wall, and the outer integuments failed to form the micropyle. The partial female sterility in this mutant

resulted from aberrant ovule development (Johns and Palmer 1982). The **ms1** mutant is male-sterile, female-fertile, but a slight female abnormality was observed (Kennell and Horner 1985). Complete or partial absence of postmeiotic cytokinesis callose walls lead to production of four-nucleate megaspores. Typically, cytokinesis lead to the formation of four uninucleate megaspores, three of which degenerate. A large number of ovules aborted; but, many underwent two successive nuclear divisions, resulting in the production of a megagametophyte bearing from 8 to 25 nuclei (Kennell and Horner 1985). Skorupska et al. (1993) reported a spontaneous mutant in soybean that alters flower development and produces apetalous male-sterile flowers. They also observed that the gynoecia were characterized by enlarged fused ovaries and exposed ovules.

Partial-sterility or semi-sterility is the condition when some of the pollen grains and (or) embryo sacs are aborted. In soybean three partial male-sterile mutants are reported: **msp** (Stelly and Palmer 1980a, 1980b), Arkansas **ms** mutant (Caviness et al. 1970); and **UPSL MS-1** (Jha and Singh 1978). All three mutants are inherited as single recessive genes. Stelly and Palmer (1982) reported that in the **msp** mutant abnormalities in the tapetum led to the degeneration of sporogenous tissue or that tapetal cells tend to precede abnormalities of associated sporogenous tissue. Meiotic

studies revealed that the sterility in **UPSL MS-1** resulted from abnormal secondary meiotic division (Jha and Singh 1978). No cytological evidence is available for the Arkansas **ms** mutant. Bernard (1989) reported the occurrence of semi-sterile and near-sterile mutants in soybean breeding populations, but he did not mention if the mutants were male or female partial-sterile.

The objectives of this study were i) to determine if the gene for partial sterility in PS-2, PS-3, and PS-4 mutants is linked to the w_4 locus and to chlorophyll-deficient mutants, CD-1 and CD-5; ii) to determine if partial-sterile mutants are allelic or if they are a new gene; iii) to study the reproductive cytology of these new mutants and compare them to reproductive development in fertile plants.

MATERIALS AND METHODS

Genetic Study

Linkage Tests The genetic materials used in this study were partial-sterile soybean mutants, PS-2, PS-3, and PS-4; two chlorophyll-deficient mutants, CD-1 and CD-5, and flower color isoline mutant w_4 in the cultivar Harosoy. PS's mutants, and CD-1, and CD-5 mutants were found in a transposon tagging study (Groose and Palmer 1987).

The CD-1 and CD-5 are chlorophyll-deficient mutants. The former segregates for green and yellow-green plants in a 3:1

ratio and the later segregates for green, yellow-green and yellow-lethal plants in an 1:2:1 ratio. The yellow-green plants have reduced vigor.

Seeds from PS's, CD-1, CD-5, and Harosoy-w_4 were sown in summer 1990 and 1991 at the Bruner Farm near Ames, Iowa. At flowering, five plants in each entry of the PS's mutants were tagged and were crossed with Harosoy-w_4, CD-1 and CD-5. Harosoy-w_4 was always used as female parent because it has white flower, recessive trait, that can be used as a morphological marker. Most of the time, CD-1 and CD-5 were used as male parent because the yellow-green plants are weak. The F_1 seed were used to generate the F_2 generation. At maturity the five tagged plants were classified for fertility based on seed set. In the F_2 generation the number of purple/white fertile and purple/white partial-sterile plants were recorded to estimate the linkage between PS's and Harosoy-w_4. The linkage estimation was calculated using the Linkage-1 computer program (Suiter et al. 1983), which uses the maximum likelihood method.

Fertile and partial-sterile F_2 plants were threshed individually in 1991, and 1992. These $F_{2:3}$ progenies were evaluated each succeeding year, and data were recorded for number of segregating and non-segregating progenies. The χ^2 test was calculated to see if the observed data fit the expected ratio.

The yellow-green F_2 plants of CD-1 and CD-5 were weak plants and set too few pods to be classified as fertile and partial-sterile. Individual F_2 plants were threshed and evaluated as $F_{2:3}$ progenies.

Allelism Tests The PS-2, PS-3, and PS-4 mutants segregated for normal and reduced number of seeds per pod in an approximate 1:1 ratio. Partial-sterile plants are easily identified at maturity due to reduced number of seeds per pod and the normal plants produce mostly two-, three-, and four-seeded pods (Groose and Palmer 1987).

Seeds from PS-2, PS-3, and PS-4 were sown in summer 1990 and 1991 at the Bruner farm, near Ames, Iowa. At flowering, five plants in each row of PS's mutants were chosen at random and tagged. Reciprocal cross pollinations for allelism test were made among PS-2, PS-3 and PS-4. The PS-2 to PS-4 tagged plants were classified at maturity for fertility/sterility due to the reduced number of seeds per pod.

The F_1 seeds were used to generate the F_2 generation; the F_2 seeds to generate the $F_{2:3}$ generation. The χ^2 test was calculated to see if the observed data fit the expected ratio.

Since semi-sterility in soybean has been associated with heterozygous translocation (Palmer and Heer 1984), reciprocal crosses were made between normal plants from PS's mutants with a standard normal chromosome soybean line 'BSR 101' to evaluate if the normal plants were homozygous for a

chromosomal abnormality or homozygous for normal chromosome structure.

Cytological Study

For cytological studies, most of the reproductive buds at different stages, were collected from tagged plants grown in the greenhouse and growth chamber. At growth chamber the temperature, and light were controlled. The temperature was 29 C during day and 26 C during night; the photoperiod was 18 hours during the first four weeks, 16 hours the next week, and 14 hours until maturity. At maturity tagged plants were classified for fertility based on seed set.

Most of the microscopy observations were done using a Zeiss Standard WL microscope. Photomicrographs were taken with a Contax Data Back Quartz D-5 camera attachment, using Kodak Technical Pan film.

Male Gametophyte Several techniques were used to evaluate fertility of pollen grains of partial-sterile plants of PS-2, PS-3, and PS-4 mutants.

Microsporogenesis For microsporogenesis, paraffin serial sections were made. Flower buds of various sizes were collected and fixed in cold FAA. After fixation these buds were dehydrated through a graded ethanol/xylene series and infiltrated with Paraplast paraffin over a minimum period of 3

days. Sections were cut on a rotary microtome at 10 μm in either longitudinal or sagittal sections, stained with safranin, and overstained with fast green solution.

Pollen viability Pollen samples were collected from each PS's tagged plant for pollen viability/fertility estimation. Pollen samples were collected at anthesis and stored in 70% ethanol at 4 C. Pollen grains were classified as normal/viable or abnormal/inviable based on the staining reaction of mature pollen grains to an iodine potassium iodide, I_2KI, solution. At the same time, measurements of pollen grain diameter were made using a 10X millimetric eyepiece.

A differential staining solution also was used to distinguish viable from nonviable pollen grains (Alexander 1969). The differential staining (Alexander 1969) was made by mixing the following:

95% alcohol - 10 ml

Malachite green - 1 ml (1% solution in 95% alcohol)

Distilled water - 50 ml

Glycerol - 25 ml

Phenol - 5 g

Chloral hydrate - 5 g

Acid fuchsin - 5 ml (1% solution in water)

Orange G - 0.5 ml (1% solution in water)

Glacial acetic acid - 2 ml

pH = 2.4-2.8

The fresh pollen grains were dusted into a drop of solution. The differentiation of aborted and nonaborted pollen grains was based upon pollen color. Aborted pollen grains were green and nonaborted pollen grains were red.

The fluorochrome reaction, FCR, method (Heslop-Harrison and Heslop-Harrison 1970) was used in an attempt to distinguish normal from abnormal pollen of partial-sterile plants. The procedure used was described by Gwyn and Stelly (1989), which is a modified fluorochrome reaction method. The modified method was prepared with mixture of two stock solutions. Stock solution I was prepared by mixing 1.75 M sucrose, 3.23 mM boric acid (H_3BO_3), 3.05 mM calcium nitrate [$Ca(NO_3)$], 3.33 mM magnesium sulfate ($MgSO_4$), 1.98 mM potassium nitrate (KNO_3), and distilled water to complete volume. Stock solution II consisted of 7.21 mM fluorescein diacetate (Sigma Chemical Co., St. Louis, MO, Lot 53F-5022) dissolved in acetone. Working solutions were prepared daily by adding 8 to 12 drops of stock solution II into 10 ml of stock solution I until the mixture became slightly milky. Fresh pollen grains were dusted into a drop of working solution and a coverslip was applied. After 2 min the slides were observed under a Zeiss Standard Fluorescent Microscope equipped with epifluorescence, barrier filter 47 25 47, and excitation filter 47 72 18.

Pollen tube germination In an attempt to observe pollen tube growth down the style to fertilize the egg cell, we designed an experiment to test for pollen tube germination. A factorial boric acid x sucrose combination was tested. The boric acid treatments were 0, 7.5, 15, and 30 ppm; sucrose treatments were 0, 5, and 10%. The best results were obtained with 7.5 ppm and 30 ppm of boric acid and 5 % and 10% sucrose. Freshly opened flowers were collected from growth chamber-grown plants early in the morning. Pollen from individual flowers was sprinkled onto the drop of boric acid x sucrose solution on each slide and allowed to grow at room temperature. After approximately one hour, the germinated pollen grains, nongerminated pollen grains and burst pollen grains were counted.

A pollen grain was considered germinated if the pollen tube had attained a length of at least 4 to 6 times the pollen grain diameter. The data were recorded as the percentage of pollen grains germinated or nongerminated in one observed microscope field.

Pollen morphology Scanning electron microscopy was used in an attempt to distinguish between normal and abnormal pollen grains from partial-sterile plants of PS's mutants based on pollen morphology and compared to normal plants. Freshly opened flowers were collected and pollen grains from individual plants were sprinkled on metallic tape which was

glued to brass discs with silver cement. A 15 nm coat of gold-palladium (20:80) was applied using a Polaron E5100 sputter coater. Observations and photographs were made using a JEOL JSM-35 scanning electron microscope at accelerating voltages of 10-20 kV.

Female Gametophyte

Megagametogenesis For paraffin serial sections, flower buds of different sizes were collected and fixed in FAA solution for at least 24 hours. A gentle vacuum was used to enhance the penetration of fixative. The gynoecia were removed, both ends were cut with a razor blade, and the gynoecia, and sometimes the ovule, were left in fixative over night. After fixation these buds were dehydrated through a graded ethanol/xylene series and infiltrated with Paraplast paraffin over a minimum period of 3 days. Sections were cut on a rotary microtome at 10 μm in either longitudinal or sagittal sections, stained with safranin, and overstained with fast green.

For resin sections flower buds of different sizes were collected and fixed in a solution of 3% glutaraldehyde and 2% paraformaldehyde in sodium cacodylate buffer (0.1 M, pH 7.2) at room temperature. The gynoecia were dissected in the fixative, place under vacuum at 15 psi (6.89 kPa) for 1 hour, and then placed in fresh fixative at 4 C overnight. After

fixation, washing was followed by three buffer rinses, postfixation in 1% osmium tetroxide (OsO_4) in same buffer for 4 hours at room temperature (22 C), and dehydrated in a graded ethanol/acetone series. The specimens were embedded in Spurr's resin (Spurr 1969), sectioned on a Reichert Ultracut E ultramicrotome at 1 to 2 μm thickness, and stained with toluidine blue.

Whole ovule clearing Ovules were dissected out of floral buds, fixed in FAA and stored for 24 hours at 4 C. After water wash, the ovules were stained in aqueous Mayer's Hemalum for 20-30 min and destained in 2% acetic acid for 10 minutes. Dehydration was carried out in an ethanol series to 100% ethanol.

The latter was gradually replaced with methyl salicylate (Stelly et al. 1984). The ovules were mounted in methyl salicylate on slides and sealed with nail polish. Slides were stored at 4 C to prevent evaporation.

The specimens also were observed under confocal scanning laser microscope (CSLM). The CSLM used in this study, Odyssey-CSLM, incorporates a DIAPHOT-NIKON inverted microscope. The specimen is illuminated by the focused light beam from an argon laser, at a excitation wavelength of 488 nm and emission wavelength at 515 nm. A NIKON PlanApo 60/1.4 oil immersion objective was used. One ovule at a time was optically sectioned at 1 μm of thickness and the microscope was

refocused 2 μm between successive images.

Ovule abortion Partial-sterile plants and normal plants from PS's mutants were harvested and brought from the field into the laboratory in 1991, 1992 and 1993. A pod-by-pod record of seed, aborted seed and aborted ovules was made for all tagged plants. Seed, seed abortions and ovule abortions within the pods were recorded as basal, middle, or apical in position. Percentage of ovule abortion was the number of ovule abortions against the total number of mature seeds, seed abortions, and ovule abortions.

RESULTS

Genetic Study

PS-2, PS-3, and PS-4 are partial-sterile mutants that segregate for normal and partial-sterile plants in an approximate ratio of 1:1 (Fig. 1) (Groose and Palmer 1987). The segregation for normal and partial-sterile plants in these mutants over two years fit the expected ratio of 1:1 (Table 1). The results of F_1 and F_2 data among normal (fertile) plants from PS-2, PS-3, and PS-4 crossed reciprocally with 'BSR 101' indicated that the normal plants in these genotypes were homozygous for normal chromosome structure and seed set since the F_1 and F_2 generations did not segregate for partial-sterile plants (Table 2).

Linkage Tests The results of F_2 linkage test of PS-2, PS-3,

and PS-4 with Harosoy-w_4 are presented in Tables 3, 5, and 7 respectively. Since PS-2, PS-3, and PS-4 segregate for normal and partial-sterile plants in a 1:1 ratio, half of F_2 population would segregate for flower color locus at 3:1 ratio but would not segregate for fertility locus. The other half would segregate for both loci, flower color and fertility. In the F_2 families segregating for both loci, segregation data, and χ^2 values were calculated for two consecutive years. For PS-2 (Table 3), in 1991 a total of 1140 F_2 plants and in 1992 a total of 352 F_2 plants were classified and for both years the segregation data fit the expected ratio of 3:3:1:1. The pooled data gave a total of 1492 F_2 plants that also fit the expected ratio. The recombination value for two years was 55% ± 0.02 and 48% ± 0.05 respectively. The results of $F_{2:3}$ progenies data (Table 4) suggest that we are dealing with two independent loci, since flower color segregates 3:1 and seed set segregates 1:1.

The results of linkage tests for PS-3 and PS-4 also indicated that the gene for partial sterility in these mutants is not linked to the flower color locus. For PS-3 (Table 5), in 1991 a total of 371 F_2 plants and in 1992 a total of 471 F_2 plants were classified and they fit the expected ratio of 3:3:1:1, that is the expected ratio for two independent loci. The $F_{2:3}$ segregating data also indicated that both loci are inherited independently (Table 6). For PS-4, over two years a

total of 836 F_2 plants were classified, 675 F_2 plants in 1991 and 161 F_2 plants in 1992 (Table 7). The results fit the expected ratio of 3:3:1:1. The $F_{2:3}$ segregation data confirmed the results obtained in F_2, that both loci were not linked (Table 8). The recombination value for PS-3 and PS-4 were around 50% indicating that the genes for flower color and fertility/partial-sterility were inherited independently.

For the linkage test between PS-2, PS-3, and PS-4 with CD-1 and CD-5, most of the time the chlorophyll-deficient mutants were used as male parent and PS's mutants were used as female parent. In the F_2 the populations segregated for plant color for the expected ratio (3:1 for CD-1; 1:2:1 for CD-5) but all the F_2 plants were fertile. The partial sterility trait was not transmitted through the female parent to the next generation (Fig. 2).

For PS-2 and PS-3 some crosses were made using CD-1 as female parent. The data from linkage test with PS-2 with CD-1 are shown in Table 9. If the genes were linked, the result would have a deviation from expected ratio. The $F_{2:3}$ segregation data showed that the gene for partial sterility was inherited independently from the gene for plant color. From a total of 153 $F_{2:3}$ families, the observed plant color data fit the expected 1:2 ratio of nonsegregating to segregating and the fertility data fit the expected 1:1 ratio. For linkage test of PS-3 with CD-1, the results showed that the plant color and

fertility loci were inherited independently (Table 10). From a total of 159 $F_{2:3}$ families, the observed plant color data fit the expected 1:2 ratio for nonsegregating to segregating families and the observed seed set data fit the expected 1:1 ratio.

The linkage tests between PS's mutants with Harosoy-w_4 and CD-1 gave us important information. The gene in these partial-sterile mutants segregated 1:1 ratio and that the partial sterility was not transmitted when the PS's were used as female parent. Transmission occurred only when PS's were used as male parent.

Allelism Tests The allelism test among PS's mutants was not possible due to the feature that the PS's mutants did not transmit the partial-sterile phenotype when they were used as female parent. Since the partial sterility was not transmitted through female parent, the observed data fit the expected 1:1 ratio.

Cytological Study

Male Gametophyte

Microsporogenesis The paraffin sections showed that there was no difference between pollen grains from normal plants and partial-sterile plants from PS-2, PS-3, and PS-4. Microsporogenesis in partial-sterile 2, 3, and 4 mutants was normal. The sporogenous cells were surrounded by the tapetal,

parietal, endothelial and epidermal cells. Microspore mother cells (MMC) were isolated by callose (Fig. 3) and after meiosis four daughter nuclei were formed (Fig. 4). These nuclei shared the same cytoplasm, and were surrounded by the original microspore mother cell plasmalemma, the callose layer, and the microspore mother cell wall. Callose was dissolved by enzymes and four individual microspores were released (Fig. 5). The mature pollen grain had wall constituted by tectum, columellae, and exine. Three colpi also were present. The mature pollen grains presented a large vegetative nucleus, and a small generative nucleus. All mature pollen grains from partial-sterile plants from PS's mutants were round, and stained red with safranin-fast green (Fig. 6).

Pollen viability The pollen viability techniques that were used to evaluate the pollen grains of PS-2, PS-3, and PS-4 did not show differences between pollen from normal plants and pollen from partial-sterile plants. With I_2KI stainability, the pollen grains from partial-sterile plants were plump, stained red-brown and were engorged with starch, presumably meaning fertile pollen grains. The pollen grains from partial-sterile plants were identical to pollen grains from normal plants (Fig. 7). No differences were observed in the measurements of the diameter of pollen grains from partial-sterile plant and normal plants. The pollen diameter was 31 μm

and 32 μm respectively.

The test with differential staining and fluorochrome reaction method (FCR) also gave the same results, that is, no differences were observed among pollen grains from partial-sterile plants. With the differential staining all pollen grains were red (Fig. 8) indicating that they were normal (nonaborted), and with FCR the background fluorescent was similar in all pollen grains (Figs. 9-10).

Pollen tube germination The pollen tube germination was higher than 80%. No difference was observed in germination of pollen from normal plants and germination of pollen from partial-sterile plants. The percentage of pollen germination for partial-sterile plants was around 85%, 82%, and 87% for PS-2, PS-3, and PS-4 respectively. The pollen tube germination was almost the same for normal plants (Table 11). Pollen tube length was more than five times the pollen diameter in both genotypes (Figs. 11-12).

Pollen morphology The morphology of pollen grains from partial-sterile plants was the same for all three mutants. Compared to the pollen grains from normal plants, no difference was observed. Pollen grains had three colpi, similar size, and shape (Fig. 13).

Female Gametophyte

Megagametogenesis The soybean ovules bend back on

themselves (campylotropous), have two integuments (bitegmic), and the megaspore forms deep in the nucellus (crassinucelate) The outer integument forms the micropyle (Prakash and Chan 1976).

Flower buds from normal and partial-sterile plants from PS's mutants were collected at anthesis, one day after anthesis, 2-3 days after anthesis, and 4-5 days after anthesis. Ovules from partial-sterile plants were dissected, sectioned and compared with the ovules from normal plants.

The embryo sac formation in soybean is Polygonum-type (Carlson and Lersten 1987). Polygonum-type embryo sacs originate from a single chalazally located megaspore that undergoes three successive mitotic divisions. The first meiotic division generated a dyad. The dyad underwent a second meiotic division resulting in a linear tetrad of megaspores. The megaspore closest to the chalazal enlarges and the three nonfunctional megaspores degenerated and were crushed by the expanding functional megaspore. The functional megaspore underwent three successive mitotic divisions. The first mitotic division resulted in two nuclei that migrated to the opposite poles and the smaller vacuoles coalesced into a large central vacuole. According to Cass et al. (1985), the vacuole is important in positioning the nuclei before subsequent mitotic divisions. Each of the two nuclei divided mitotically two more times, resulting in a seven-celled megagametophyte.

Ovules at anthesis showed normal ovules, with the egg apparatus at micropylar end and polar nuclei or secondary endosperm nucleus in close proximity to the egg apparatus (Figs. 14-17). The antipodals were degenerated as was expected. The central cell was filled with starch grains. Sometimes we observed the sperm cell close to the nucleolus of the fused polar nuclei (Fig. 16).

Ovules two- three days after anthesis showed the zygote and more advanced stages of embryo development, such as young proembryo and free nuclear endosperm. Ovules five days after anthesis started to show differences in size and were both small and large ovules. The small one was not fertilized and the egg apparatus and secondary endosperm nuclei could be seen and the large one was at proembryo stage with free nuclear endosperm.

Ovaries five days after anthesis from partial-sterile plants, had aborted ovules with the egg apparatus and polar nuclei/secondary endosperm nucleus intact suggesting that the double fertilization did not happen (Figs. 18-25). In serial sections, pollen tubes were observed entering into the micropyle indicating that the ovule abortion was not due to failure of pollen tube germination. In nonaborted ovules, five days after anthesis, proembryo, and globular stages were observed (Figs. 26-29).

Ovule abortion The percentage of ovule abortion for

normal and partial-sterile plants from PS-2, PS-3, and PS-4 mutants over three years is presented in the Table 12. The percentage of ovule abortion was similar in two- and three-ovule pods among the three partial-sterile mutants.

DISCUSSION

Genetic Study

Linkage Tests PS-2, PS-3, and PS-4 were recovered from the Asgrow mutable line. This soybean line is suspected to contain a transposable element at the w_4 locus. The linkage test between the gene for partial-sterility in the PS's mutants with Harosoy-w_4 indicated the both loci were inherited independently, that means that they were not linked.

Since transposable elements transpose from one chromosome to another or within the same chromosome, some mechanism may be involved to fate the movement of the transposon. According to Cone and Schimdt (1987), several lines of evidence indicated that transpositions often occurs to sites that are linked to the original site of the element. McClintock (1963) working with **Spm** found that 33% of the transposition were to sites linked to **a1**. Peterson (1970) found that transposition of **En** away from **a1-m** (pa-pu) were to linked sites 25% of the time, and that there was a preference for transposition to sites 6-20 map units from **a1-m**. Transposition seemed to occur to sites both proximal and distal to the original site

insertion. Van Schaik and Brink (1958) also observed that the transposition of **Ac** from P-vv was to linked sites 67% of the time and they concluded that transposition seems to occur to sites distal on the chromosome, whereas transpositions of **Ac** from **bz1-m2** appeared to occur in both directions, distal and proximal sites. Epperson and Clegg (1992) working with unstable white flower color genes and their derivatives in morning glory (*Ipomoea purpurea*) found a new mutant, double flowers. This new gene (**D/d**) was a single recessive gene and was unlinked or loosely linked to the **A/a** locus responsible for white flower.

Palmer et al.(1989) working with chlorophyll-deficient mutants (CD-1, CD-2 and CD-3) and necrotic root mutants (NR-1, NR-2, and NR-3) obtained from the same population that included PS's, found that these mutants were not linked to the w_4 locus. The partial-sterile 1 mutant, that also was recovered from the same population as PS-2, PS-3, and PS-4 was not linked to the w_4 locus (Pereira, Lersten, and Palmer, unpublished). The evidence suggests that in the Asgrow mutable line, transposition does not occur to linked sites, thus explaining the independence of the loci. No molecular evidence is available to support that the w_4 locus has a transposable element insertion, therefore our results need to be confirmed molecularly.

Linkage tests between PS's mutants with CD-1 showed that

the gene in chlorophyll-deficient 1 was not linked to the gene that causes partial-sterility in PS-2 and PS-3. The transposable element in w_4 line transposes more than 50 centiMorgans. The PS-1 mutant was recovered from the same population as PS-2, PS-3 and CD-1 and was not linked to the CD-1 gene (Pereira, Lersten, and Palmer, unpublished). The linkage test of CD-1 with PS-4 and CD-5 with PS-2, PS-3, and PS-4 was not possible because the PS mutants were used as female parent. All F_2 plants were normal for seed set and segregated for plant color. This is additional evidence that the gene for partial sterility was not transmitted through the female parent in these PS's mutants.

The linkage tests gave information that the gene in the PS's mutants is inherited in a 1:1 ratio; all F_2 normal plants did not segregate in the $F_{2:3}$ generation confirming normal chromosome structure.

Allelism Tests Reciprocal crosses were made between PS-2, PS-3, and PS-4. Due to the nontransmission of partial sterility to the next generation when PS-2, PS-3, and PS-4 were used as female parent, the data from allelism tests behave as if was a selfing generation instead of hybrid origin. That means, the F_2 data would segregate in a 1:1 ratio. The observed data fit the expected 1:1 ratio.

Partial sterility has been reported in maize by Singleton and Mangelsdorf (1940), Clark (1942), Nelson and Clary (1952).

These authors reported the occurrence of three independent lethal ovule factors **lo**, **lo1** and **lo2**. These lethal factors have the same behavior as the gene in PS-2, PS-3, and PS-4: they were inherited in a 1:1 ratio; all pollen grains were normal; and the trait was not transmitted when used as female parent. Basically the **lo** factor acts before fertilization and the recessive genotype rarely is capable of being fertilized. Heterozygous plants for **lo** (**lo/+**) are as vigorous as those with constitution (**+/+**) and there was no noticeable difference in time of flowering. Homozygous recessive plants (**lolo**) were not obtained. The **lo** locus was located to chromosome 4 and no deficiencies were observed in chromosome 4 and the other chromosomes were apparently normal. The **lo** factor was closely linked to the **su** locus in maize (Singleton and Mangelsdorf 1940). The lethal factor described by Clark (1942), behaves similarly to the **lo** factor, since it had 50% ovule abortion and normal pollen, but it was not linked to the **su** locus on chromosome 4. Van Horn and Nelson (1969) reported a lethal ovule factor linked to the **wx** locus.

Redei (1965) described a mutation with gametophytic expression, **Gf**, in *Arabidopsis*. This mutation was not transmitted through the egg, was inherited relatively normally through the sperm, produced abnormal ratios of linked markers, and seemed to segregate in a non-Mendelian way. Based on these results, the author concluded that the trait was an ovule-

abortion or egg-lethality factor. In the literature there are examples of gene that affect the female gamete such as gamete aborter, Ge^c. However, Ge^c affects the male and female gametes equally; acts only in heterozygous combinations with a specific allele, Ge^p; and behaves as if incompletely penetrant (Rick 1966).

Our results indicated that the gene in PS-2, PS-3, and PS-4 behaves similarly to the lethal ovule mutants in maize since we had no transmission through the female. We had ovule abortion. All pollen grains from partial-sterile plants were normal.

Cytological Study

Male Gametophyte

Microsporogenesis Partial-sterile plants from PS-2, PS-3, and PS-4 had normal microsporogenesis resulting in normal pollen grains. The microsporogenesis start with the division of a diploid sporophyte cell, giving rise to the tapetal initial and sporogenous tissue. During the formation of pollen grains a sequence of events leads to the formation of pollen grains. Any disruption in one of these events can result in pollen sterility (McCormick 1993). Graybosch and Palmer (1988) and Palmer et al. (1992) summarized the male- sterile mutants that were found in soybean. The most common type of reproductive mutations observed in soybean are those that

induce male sterility. The high frequency of occurrence of male-sterile mutations indicated that a number of genes influence the process of microgametogenesis and microsporogenesis (Graybosch and Palmer 1988).

Since the microsporogenesis resulted in mature pollen grains with large vegetative nucleus, small generative cell and stained red with safranin-fast green, meaning viable/fertile pollen grains, we assumed that the microsporogenesis was completely normal in the PS's mutants.

Pollen viability The viability of pollen grains from partial-sterile plants from PS-2, PS-3, and PS-4 was assessed by using different stains. The I_2KI staining is used to distinguish normal from abnormal pollen grains in male sterile mutants. Benavente et al. (1989) working with a male-sterile, female-sterile soybean mutant, observed that pollen grains in fertile plants stained densely and were well rounded, with intact cytoplasm, walls, and colpi, whereas sterile plants had lightly staining pollen grains, often with collapsed cytoplasm. Our results with pollen grains from partial-sterile plants from PS-2, PS-3, and PS-4 mutants stained with I_2KI showed pollen grains densely stained, rounded, engorged with starch, indicating fertile pollen grains.

The differential staining (Alexander 1969) used to distinguish aborted from nonaborted pollen grains, showed that the pollen grains from partial-sterile mutants (PS-2, PS-3,

and PS-4) were viable/ fertile since they stained an intense red. The pollen walls were green color and the cytoplasm was red, meaning viable pollen grains. Pollen from normal plants also stained an intense red. According to Alexander (1969), the aborted pollen grains are stained green because only the pollen wall is stained with Malachite green. In our study, we did not observe pollen grains stained green. These results confirm that the pollen grains from partial-sterile mutants are fertile/viable.

According to Barrow (1983) most of the stains discriminate between living and dead pollen, and among pollen grains of known male-sterile and male-fertile types, but were not reliably effective in the classification of functional pollen grains with reduced viability and thus tended to overestimate sporophytic fertility. The viability of the vegetative cell of the male gametophyte is correlated with the state of the plasmalemma; if this shows normal permeability, the cell is likely to be viable. Heslop-Harrison and Heslop-Harrison (1970), developed a technique to evaluate the membrane integrity of the pollen grains, based on the fluorescence of the pollen grains after exposure to fluorescent diacetate (FCR).

The FCR method modified by Gwyn and Stelly (1989) proved to be an effective tool for the evaluation of pollen viability/fertility in a manner that provides a phenotype that

accurately distinguished in cotton chromosome translocation heterozygotes from homozygotes or normals (Gwyn and Stelly, 1989). Using the FCR method, Gwyn and Stelly (1989) were able to distinguish fertile pollen grains from sterile pollen grains. The former were the largest, fully engorged with starch, fluoresced bright luminescence green, and were considered fully viable (fertile). The later, harbored an array of pollen types, including pollen grains that: (i) were small but fluoresced brightly, (ii) were large or small and fluoresced dimly, (iii) fluoresced unevenly with lighter and darker areas, and (iv) did not fluoresce at all. With FCR method the pollen grains from partial-sterile plants from PS's mutants were not different from pollen grains from normal plants, all of them had the same size and had the same fluorescent bright luminescent green background. Based on these observations, we concluded that the pollen grains from partial-sterile plants from PS's were fully fertile.

Pollen germination Pollen germination tests have been reported to provide reasonable estimates of pollen fertility. During growth of the pollen tube toward the ovule in soybean, the generative cell divides and forms two male gametes, the sperm cells (Carlson and Lersten 1987). Finally the pollen tube grows through the micropyle of the ovule, and enters the filiform apparatus of the degenerated synergids. The pollen tube tip bursts and releases the two sperm cells. One sperm

cell fuses with the egg cell and forms the diploid zygote, the other sperm cell fuses with the secondary nucleus forming the primary endosperm nuclei. This is called double fertilization.

Since we had good response with viability test of pollen grains from partial-sterile plants, the next step was to observe if the pollen grains were able to grow down the style to fertilize the egg cell. Pollen-tube germination was very good, confirming once again that the pollen grains from partial-sterile plants were fully viable/fertile. Chen et al. (1987) working with male-sterile **ms1** soybean observed that coenocytic microspore tubes rarely were observed in gynoecia from male-sterile plants or in gynoecia from male-fertile plants that had been artificially cross-pollinated with **ms1ms1** plants. In serial sections of ovules one day after anthesis, we observed trace of pollen tubes entering into the micropyle.

Pollen morphology The morphology of pollen grains from PS's mutants was observed using scanning electron microscopy (SEM) in an attempt to distinguish between normal and abnormal pollen grains from PS's mutants.

The SEM observations showed that all pollen grains were similar, with same shape, size, and three colpi.

Since PS-2, PS-3, and PS-4 mutants are partial-sterile, and we thought that the pollen grains would show a degree of abnormality, we tested the pollen grains from PS's mutants with several techniques. All methodologies that we used failed

to show differences between pollen grains from partial-sterile plants and pollen grains from normal plants. These results indicated that the partial sterility in PS-2, PS-3, and PS-4 is not due to abnormality in the pollen grains. The pollen grains from PS-2, PS-3, and PS-4 mutant sare completely fertile/viable.

Female Gametophyte

Megagametogenesis The megagametogenesis in ovules of PS-2, PS-3, and PS-4 mutants were completely normal. There were no differences in development between ovules from partial-sterile plants and normal plants in regard to embryo sac formation. Megasporogenesis and megagametogenesis has been described in soybean using light and electron microscopy (Carlson and Lersten 1987; Kennell and Horner 1985; Folson and Cass 1990, 1992; Chamberlin et al. 1993).

Ovules from normal and partial-sterile plants from PS's mutants had normal megagametogenesis resulting in a megagametophyte with six uninucleate cells and one binucleate cell. This is consistent with Polygonum-type development (Kennell and Horner 1985; Carlson and Lersten 1987). The position of polar nuclei were consistent, positioned close or at proximity to the egg apparatus (Maheshwari 1950; Kennell and Horner 1985; Carlson and Lersten 1987). Other features as central cell filled with starch grains also was observed in

ovules from normal and partial-sterile plants.

Double fertilization is represented by the fusion of one sperm cell with the egg cell and the second sperm cell with the secondary endosperm nucleus. According to Chamberlin et al. (1993), immediately after fertilization, the nucleoplasm of the primary endosperm nucleus was homogeneous and had no inclusions except for two nucleoli of different sizes. The smaller nucleolus was in the lobe of the primary endosperm nucleus and represented the fused sperm nuclei. In subsequent stages, the primary endosperm nucleus was spherical and had a single large nucleolus. Ovules from flowers at anthesis, from normal and partial-sterile plants, showed these features described by Chamberlin et al. (1993).

Comparison between ovaries 5 DAP, from normal plants and from partial-sterile plants, showed that ovaries from normal plants had ovules almost at same stage of embryo development. However, ovaries from partial-sterile plants had two different embryo/ovule sizes, large and small. The large one, fertilized, showed proembryo at different cell stage and free nuclear endosperm. The small one was unfertilized, usually the egg apparatus and secondary endosperm nucleus could be seen at micropylar end. Sometimes the degeneration was advanced and only remnants of the egg apparatus and secondary endosperm nucleus could be detected. Contolini and Menzel (1987) working with two heterozygous translocated cotton lines reported a

high percentage of aborted ovules with intact synergids and polar nuclei, indicating that failure may occur during fertilization. Mogensen (1975), also reported that in *Quercus* (Fabaceae) 45% of the ovule abortion in this species is due to lack of fertilization, both synergids remained full and intact throughout the life of the ovule and the egg collapsed very early. Burbidge and James (1991) observed reduction in seed formation after self-pollination compared to that after cross-pollination in *Stylidium*. They concluded that recessive lethal factors induced ovule/seed abortion after entry of the pollen tube into the micropyle of the ovule.

The lethal factors reported in maize were due to the abortion of ovules that either were aborted before fertilization or were unable to be fertilized (Clark 1942; Singleton and Mangelsdorf 1942; Nelson and Clary 1952)

Ovule abortion The percentage of ovule abortion in the partial-sterile plants was different from the percentage of ovule abortion in normal plants, indicating that the ovule abortion in PS's mutants is caused by genetic factors. According to Meinke and Sussex (1979) embryogeny is one of the few developmental pathways in higher plants that is relatively insensitive to the environmental factors, therefore it is primarily under genetic control. The distribution of ovule abortion in pods in PS's mutants was at random, suggesting that the effect of mutant allele was not either on pollen

germination or pollen-tube growth and it was based on the distribution of mutant ovules within the ovary. Segregation distorters and other factors affecting megagametogenesis are rare in higher plants (Redei 1965) and they appear to alter segregation ratios uniformly throughout the ovary (Meinke 1982). Palmer and Herr (1984) observed random distribution of the ovule abortions in a heterozygous chromosome translocation in soybean. Meinke (1982) working with *Arabidopsis thaliana*, observed 60% of the aborted seeds in 79A and 124D mutants were located in the top half of heterozygous siliques. The deviation from the expected (25%) random distribution of aborted seeds indicated that the non-random distribution of the aborted seeds in these mutants was more likely caused by a disruption of either pollen development, pollen germination, or pollen-tube growth.

Clark (1942) reported that the lethal factor, **lo**, usually causes the abortion of ovules carrying it due to abortion before fertilization or some abnormality that prevents the fertilization. In PS-2, PS-3, and PS-4 mutants we had lack of fertilization even though the embryo sac had normal development. Based on these results, we believe that the partial sterility in PS-2, PS-3, and PS-4 mutants was due to a gene that resembles the lethal factor in maize, that means that the ovule carrying the gene was fated to die. Pollen grains from the PS-2, PS-3, and PS-4 mutants were fertile.

Table 1. Number of normal (N) and partial-sterile (PS) plants from self-pollination of partial-sterile soybean mutants (Summer 1990, 1991, Ames, Iowa)

Family	1990	1991	Total	χ^2	P
	N:PS	N:PS	N:PS	(1:1)	
PS-2	25:29	40:39	65:68	0.07	0.79
PS-3	28:22	31:34	59:56	0.08	0.78
PS-4	29:36	59:56	88:92	0.09	0.76

Table 2. Number of F_1 and F_2 soybean plants from reciprocal crosses of normal plants of PS-2, PS-3, and PS-4 with 'BSR 101'

Cross	F_1		F_2	
	Number of plants	Phenotype	Number of plants	Phenotype
'BSR 101' X PS-2	16	Normal	2400	Normal
'BSR 101' x PS-3	14	Normal	2100	Normal
'BSR 101' x PS-4	27	Normal	4050	Normal
PS-2 x 'BSR 101'	14	Normal	2100	Normal
PS-3 x 'BSR 101'	11	Normal	1650	Normal
PS-4 x 'BSR 101'	15	Normal	2250	Normal

Table 3. Number of F_2 plants from linkage test from crosses between Harosoy-w_4 as female parent with soybean partial-sterile (PS-2) as male parent

Phenotypes	Year		Total
	1991	1992	
Purple normal	447	131	578
Purple partial-sterile	406	139	545
White normal	132	43	175
White partial-sterile	155	39	194
Total	1140	352	1492
χ^2(3:3:1:1)	3.53	0.97	1.97
P	0.32	0.81	0.58
%R	55.00	48.00	53.00
SE	±2.00	±5.00	±2.00
Purple:white	853:287	270:82	1123:369
χ^2 (3:1)	0.02	0.54	0.06
P	0.89	0.46	0.81
Normal:partial-sterile	579:561	174:178	753:739
χ^2 (1:1)	0.28	0.04	0.13
P	0.60	0.84	0.72

Table 4. Number of $F_{2:3}$ progenies from crosses between Harosoy-w_4 as female parent with soybean partial-sterile mutant 2 (PS-2) as male parent

	Flower color		
	Purple	Purple and white	White
Normal	22	55	20
Normal and partial-sterile	24	44	36
Purple:(purple and white):white		46:99:56	
χ^2 (1:2:1)		0.83	
P		0.66	
Normal:(normal and partial-sterile)		97:104	
χ^2 (1:1)		0.18	
P		0.67	

Table 5. Number of F_2 plants from linkage test from crosses between Harosoy-w_4 as female parent with soybean partial-sterile 3 mutant (PS-3) as parent male

Phenotypes	Year		Total
	1991	1992	
Purple normal	134	179	313
Purple partial-sterile	147	171	318
White normal	48	71	119
White partial-sterile	42	50	92
Total	371	471	842
χ^2(3:3:1:1)	0.86	4.04	3.51
P	0.83	0.26	0.32
%R	46.00	47.00	47.00
SE	±5.00	±3.00	±3.00
Purple:white	281:90	350:131	631:211
χ^2 (3:1)	0.11	0.10	0.002
P	0.74	0.75	0.96
Normal:partial-sterile	182:189	250:221	432:410
χ^2 (1:1)	0.13	1.79	0.57
P	0.72	0.18	0.45

Table 6. Number of $F_{2:3}$ progenies from crosses between Harosoy-w_4 as female parent with soybean partial-sterile mutant 3 (PS-3) as male parent

	Flower color		
	Purple	Purple and white	White
Normal	21	42	29
Normal and partial-sterile	29	49	25
Purple:(purple and white):white		50:91:54	
χ^2 (1:2:1)		0.99	
P		0.61	
Normal:(normal and partial-sterile:		92:103	
χ^2 (1:1)		0.62	
P		0.43	

Table 7. Number of F_2 plants from linkage test from crosses between Harosoy-w_4 as female parent with soybean partial-sterile 4 mutant (PS-4) as male parent

Phenotypes	Year		Total
	1991	1992	
Purple normal	250	61	311
Purple partial-sterile	259	60	319
White normal	86	21	107
White partial-sterile	80	19	99
Total	675	161	836
χ^2(3:3:1:1)	0.36	0.05	0.41
P	0.95	0.98	0.94
%R	49.00	49.00	49.00
SE	±3.00	±6.00	±3.00
Purple:white	509:166	121:40	630:206
χ^2 (3:1)	0.06	0.002	0.06
P	0.81	0.96	0.81
Normal:partial-sterile	336:339	82:79	418:418
χ^2 (1:1)	0.01	0.06	0.001
P	0.92	0.81	0.99

Table 8. Number of $F_{2:3}$ progenies from crosses between Harosoy-w_4 as female parent with soybean partial-sterile mutant 4 (PS-4) as male parent

	Flower color		
	Purple	Purple and white	White
Normal	25	47	26
Normal and partial-sterile	26	52	24
Purple:(purple and white):white		51:99:50	
χ^2 (1:2:1)		0.03	
P		0.98	
Normal:(normal and partial-sterile)		98:102	
χ^2 (1:1)		0.08	
P		0.78	

Table 9. Number of $F_{2:3}$ progenies from crosses between CD-1 as female parent with soybean partial-sterile mutant 2 (PS-2) as male parent

	Plant color	
	Green	Green and yellow green
Normal	28	56
Normal and partial-sterile	20	49
Green:(green and yellow green)		48:105
χ^2 (1:2)		0.26
P		0.60
Normal:(normal and partial-sterile)		84:69
χ^2 (1:1)		1.47
P		0.25

Table 10. Number of $F_{2:3}$ progenies from crosses between CD-1 as female parent with soybean partial-sterile mutant 3 (PS-3) as male parent

	Plant color	
	Green	Green and yellow green
Normal	29	59
Normal and partial-sterile	22	49
Green:(green and yellow green)		51:108
χ^2 (1:2)		0.11
P		0.74
Normal:(normal and partial-sterile)		88:71
χ^2 (1:1)		1.82
P		0.18

Table 11. Percentage pollen germination from partial-sterile mutants (PS-2, PS-3, and PS-4) in two combinations of sucrose x boric acid solutions

Plant	5% Sucrose and 7.5 ppm boric acid	10% Sucrose and 30 ppm boric acid
PS-2		
Normal	89	86
Partial-sterile	87	83
PS-3		
Normal	83	80
Partial-sterile	81	83
PS-4		
Normal	86	87
Partial-sterile	88	86

Table 12. Percentage of ovule abortion in three partial-sterile soybean mutants, Ames, Iowa.

	Number of plants			Percentage of ovule abortion in pods with 2 seeds			Percentage of ovule abortion in pods with 3 seeds		
	1990	1991	1992	1990	1991	1992	1990	1991	1992
PS-2									
Normal	14	32	n.d	17.8	21.3	n.d	25.1	17.2	n.d
Partial-sterile	16	18	5	33.6	32.1	31.0	43.9	43.1	44.0
PS-3									
Normal	17	12	n.d	22.0	19.8	n.d	27.0	17.2	n.d
Partial-sterile	8	12	5	31.1	32.9	39.0	46.5	43.2	47.0
PS-4									
Normal	5	17	n.d	15.5	15.7	n.d	19.6	14.0	n.d
Partial-sterile	5	8	5	29.5	35.6	38.0	46.6	41.7	42.0

n.d: no data

♀ Gametes	♂ Gametes	
	B	**b**
B	BB (Normal)	Bb (PS-2)
b	ABORTED	ABORTED

⇓

1 (N) : 1 (PS)

Figure 1. Self-pollination of partial-sterile plant from PS-2 mutant

Assumptions:

1) **b**: single recessive gene for partial sterility in PS-2 (**Bb**) (or PS-3, or PS-4) mutant, transmitted only through the male parent.

♀ Gametes	♂ Gametes	
	GB	**gB**
GB	GGBB (Green and Normal)	GgBB (Yellow-green and Normal)
Gb	ABORTED	ABORTED

Figure 2. Scheme of self-pollination for a cross between partial-sterile plant from PS-2 used as female with CD-1 as male parent.

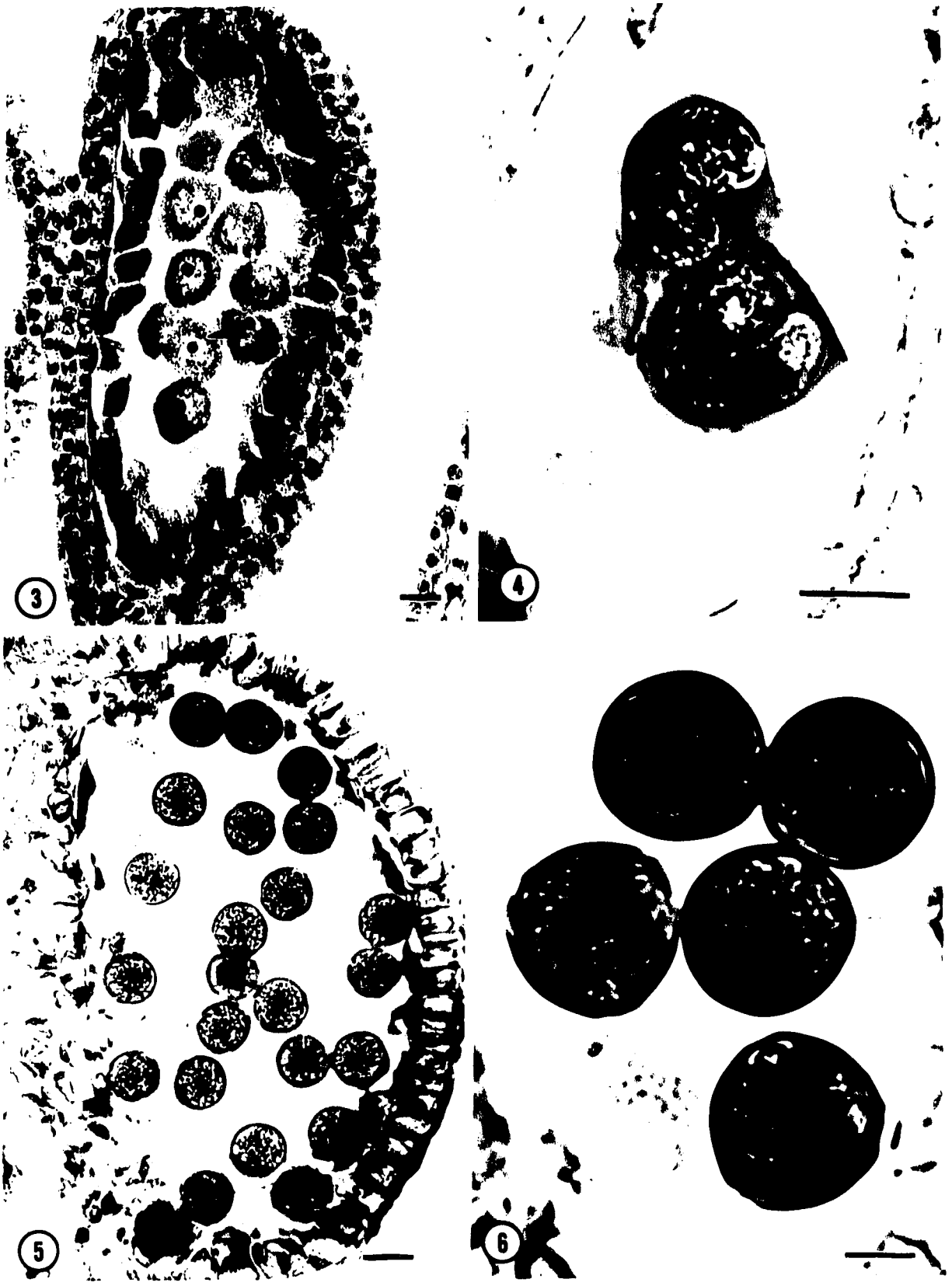
Assumptions:

1) PS-2 is green and partial-sterile (GGBb)

2) CD1 is yellow-green and normal (GgBB)

Figs. 3-6. Microsporogenesis in partial-sterile plants from PS's mutants.

3. Meiotic stage. MMC nuclei (arrow) are enlarged. Bar = 10μm
4. Tetrad stage. Bar = 10μm
5. Anther with mature pollen grains. Bar = 25μm
6. Mature pollen grains filled with starch. Bar = 10μm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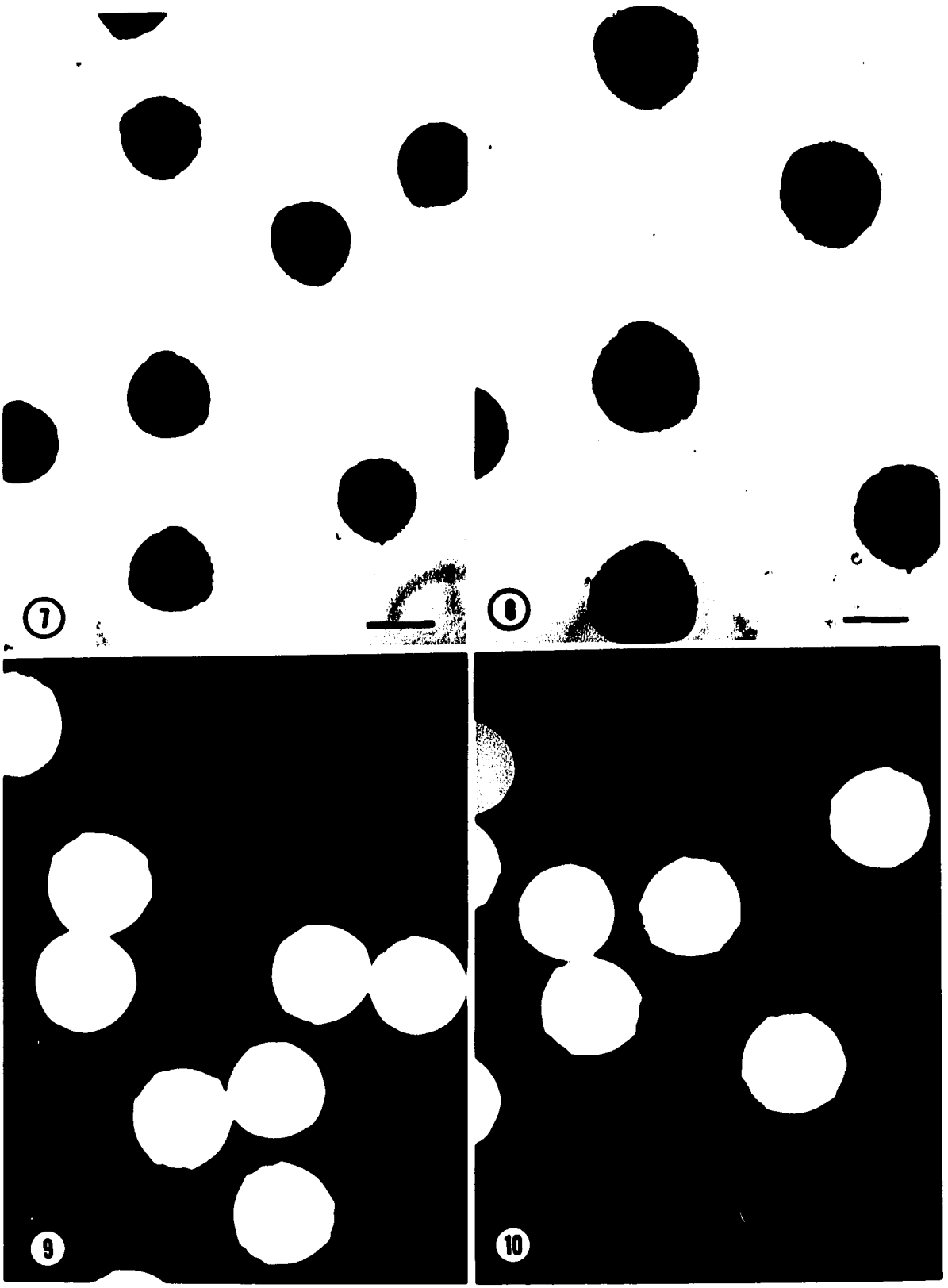
Figs. 7-10. Pollen grains from partial-sterile plants from PS-2, PS-3, and PS-4 soybean mutants stained with different techniques.

7. Pollen grains from partial-sterile plants stained with I_2KI, showing pollen grains engorged with starch. Bar = 20μm

8. Pollen grains from partial-sterile plants stained with Malachite green, fucsin acid, and Orange G (differential staining). Bar = 10μm

9-10. Comparison between pollen grains from normal plants (Fig.9) and pollen grains from partial-sterile plants (Fig. 10) stained with FCR. Both genotypes showed the same reaction. Bar = 20μm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Figs. 11-12. Comparison between pollen grains from normal plants (Fig. 11) and pollen grains from partial-sterile plants (Fig. 12) from PS's mutants germinated in boric acid x sucrose solution. Both genotypes had the same response to pollen-tube germination test. Bar = 5μm

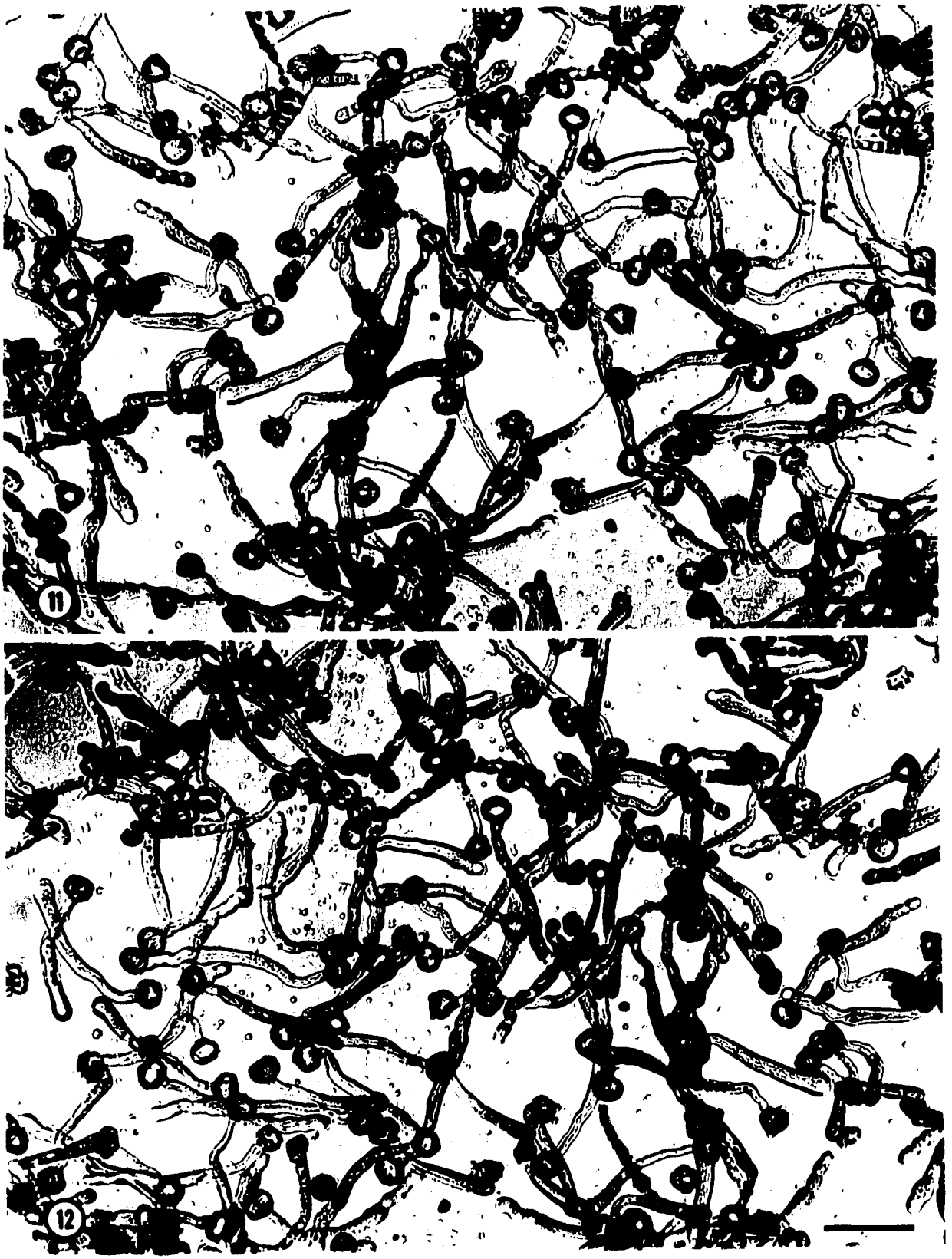
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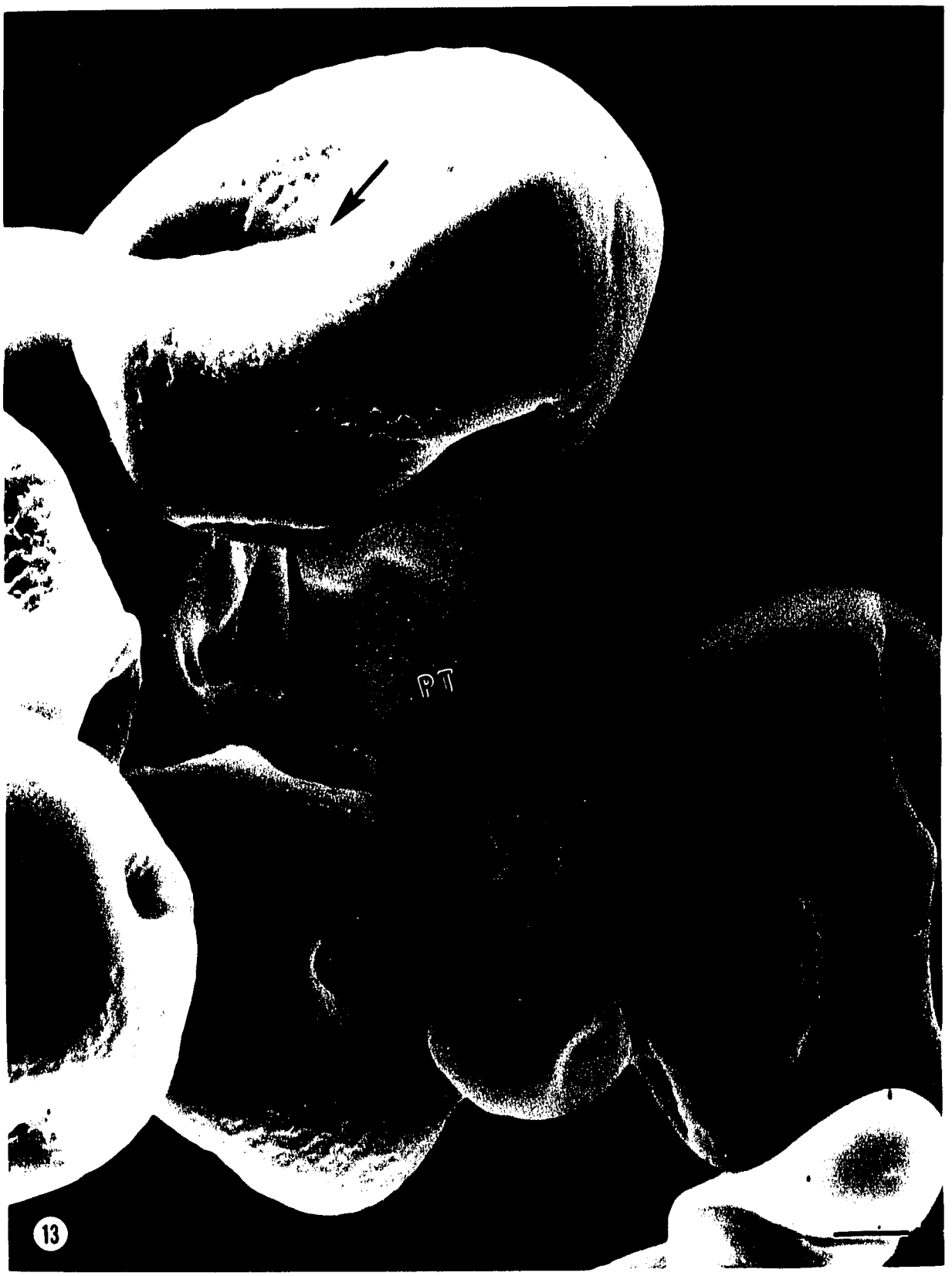
Fig. 13. Pollen grain from PS's mutants showing the colpi (arrow) and pollen tube (PT). Bar = 5μm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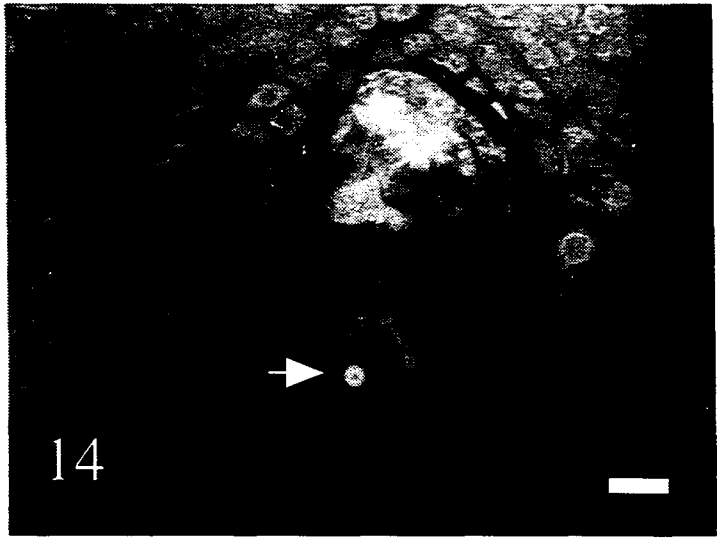
Figs. 14-17. Megagametophyte in ovules from PS's soybean mutants.

14. Secondary endosperm nucleus (arrow) in the central cell. Bar = 10μm

15. Egg cell (arrow) at micropylar end. Bar = 10μm

16. Secondary endosperm nucleus showing the sperm nucleus (arrow). Bar = 10μm

17. Synergid (arrow) micropylar end. Bar = 10μm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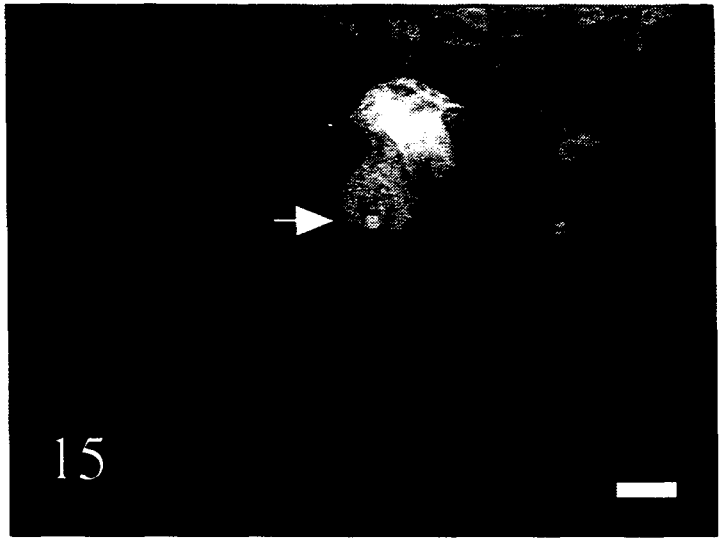
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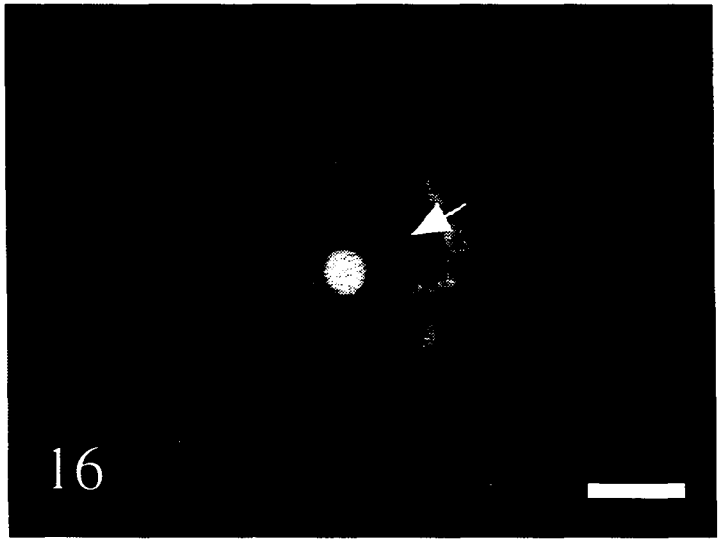
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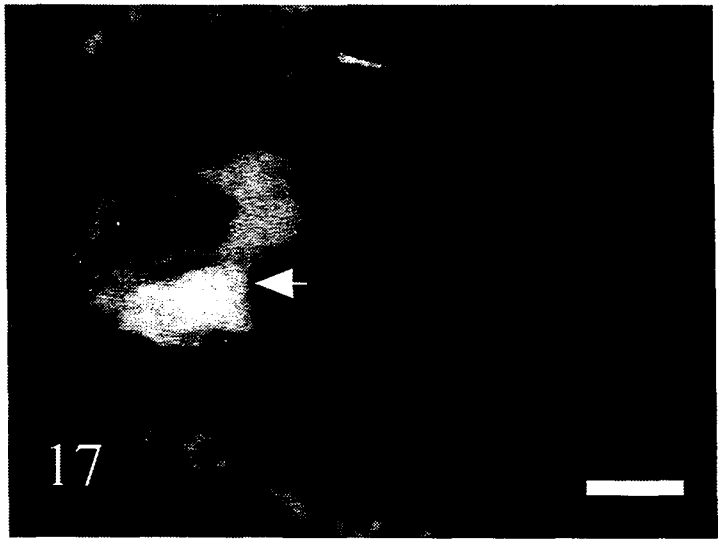
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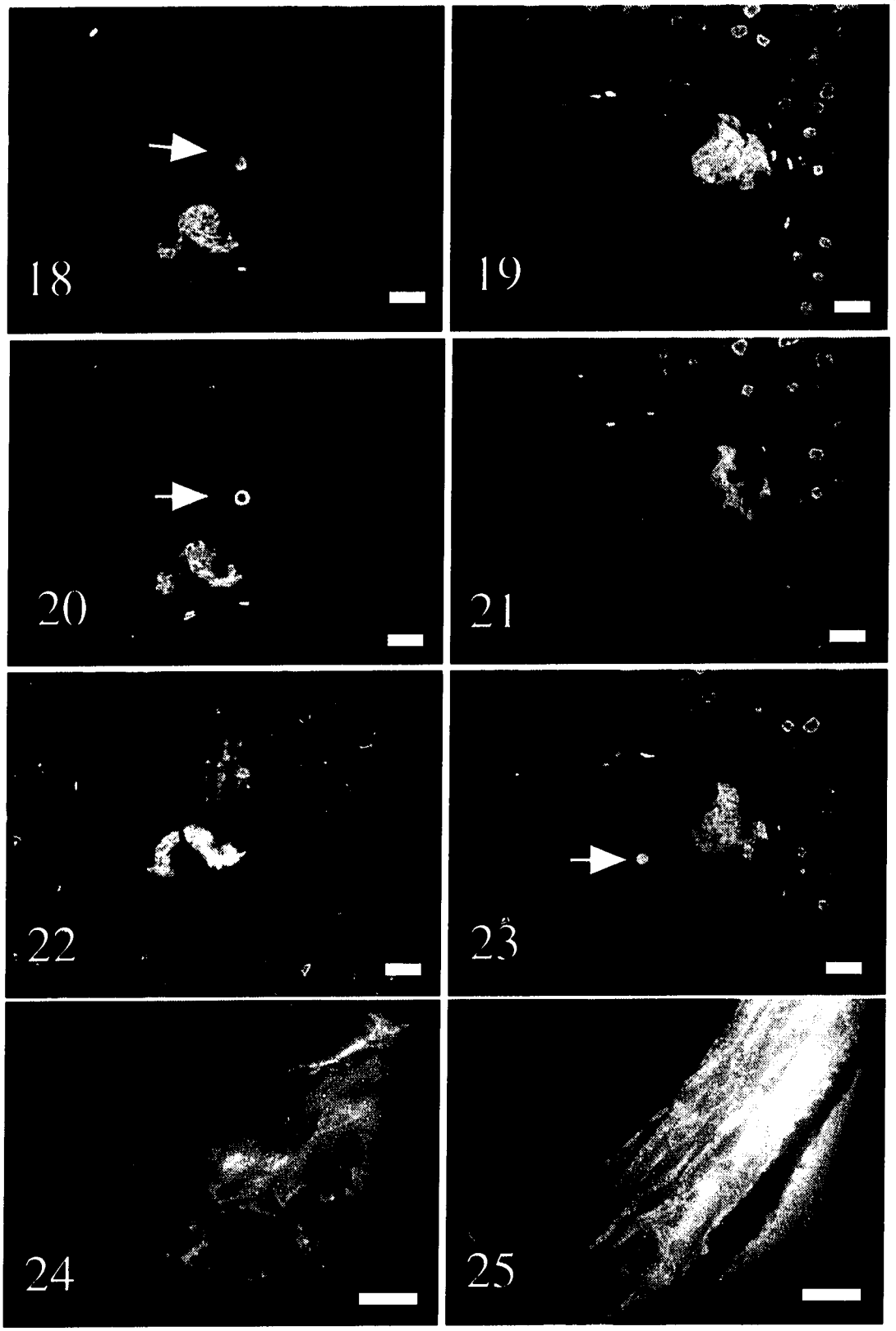
Figs. 18-23. Optical sections of a degenerated ovule from PS's mutants showing the egg apparatus and secondary endosperm nucleus intact. Note the position of secondary endosperm (arrow) close to the egg cell. Bar = 10μm

Figs. 24-25. Late degeneration in ovules from PS's mutants showing traces of cells in the the central cell. Bar = 10μm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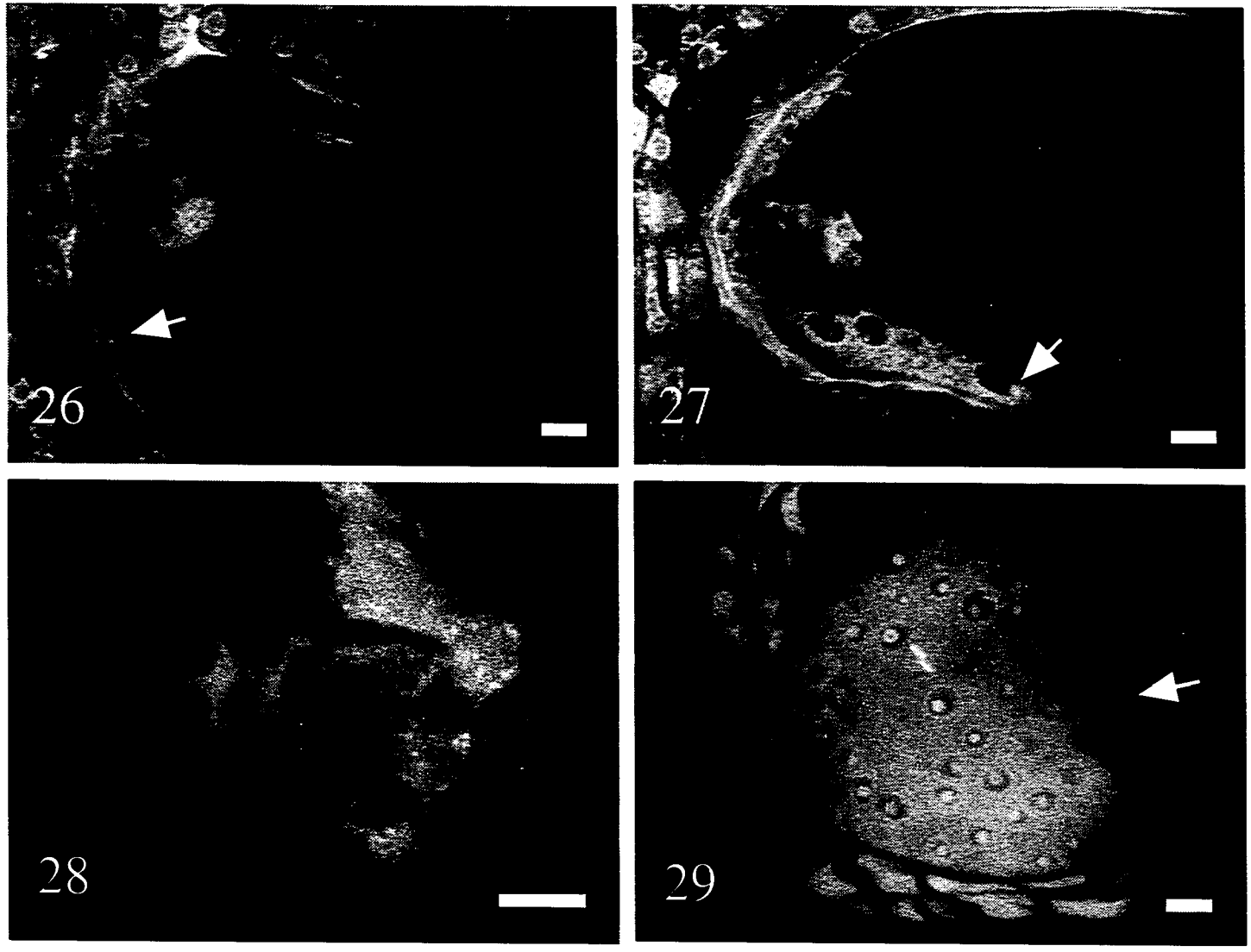
Figs. 26-28. Different stages of proembryo development in ovaries from partial-sterile plants from PS's mutants. Note the free nuclear endosperm (arrow). Bar = 10μm

Fig. 29. Embryo at globular stage (arrow) at micropylar end. Bar = 10μm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GENERAL CONCLUSIONS

Four partial-sterile mutants, PS-1, PS-2, PS-3, and PS-4 were recovered in a soybean population that is suspected to have a transposable element insertion at the w_4 locus. The main objectives were to study the genetics and cytology of the partial-sterile soybean mutants.

The linkage studies for PS-1, PS-2, PS-3, and PS-4 with the w_4 locus indicated that the partial-sterile mutants were not linked to the w_4 locus. Others mutants, CD-1, CD-2, CD-3, and necrotic root mutants, recovered from the same population as PS's were not linked to the w_4 locus (Palmer et al., 1989). These results suggested that the transposition in the Asgrow mutable line was not to linked sites. Since no molecular evidence is available to support the hypothesis that the w_4 locus has a transposable element insertion, our results need to be confirmed molecularly.

The linkage studies between PS-1, PS-2, and PS-3, with CD-1 showed that the gene in the PS-1, PS-2, and PS-3 was not linked to the gene in chlorophyll-deficient 1 mutant. The F_3 data indicated the independence for the two loci; fertility and plant color.

The linkage studies confirmed the inheritance of the PS's mutants. The PS-1 mutant was inherited as a single recessive gene, explaining its true breeding behavior. PS-2, PS-3, and PS-4 mutants were inherited in a 1:1 ratio,

segregating normal and partial-sterile plants.

Additional information obtained from this research was that the fertile plants from PS-2, PS-3, and PS-4 were homozygous for normal chromosome structure, since the F_1 and F_2 plants from reciprocal crosses between normal/fertile plants from PS's with 'BSR 101' were normal for seed set. The linkage study also indicated that partial-sterility was not transmitted through the female parent when PS-2, PS-3, and PS-4 were used as female parents.

The allelism test had the objective to determine if the gene in the four partial-sterile mutants was a new gene or if they were the same gene. Reciprocal crosses were made and F_2 and F_3 plants were evaluated. The gene in PS-1 was not allelic to the gene in PS-2, PS-3, and PS-4. This result was expected since the gene in PS-1 was maintained as a true breeding genotype. Partial-sterile plants of PS-2, PS-3, and PS-4 upon self-pollination segregated for normal and partial-sterile plants in a 1:1 ratio. A true breeding genotype for PS-2, or PS-3, or PS-4 was not obtained. From allelism tests double partial-sterile mutants were recovered that had more severe reduction on seed set, because it had combined genes from partial sterility of PS-1 with PS-2 or PS-3 or PS-4. The allelism test between PS-2, PS-3 and PS-4 was not possible because the partial sterility in these mutants was not transmitted to the next generation when one of them was used

as female parent. These data supported the results obtained in linkage test between PS's mutants with CD-1 and CD-5 that partial sterility was not transmitted when the PS's mutants were used as female parent.

From cytological studies, we observed complete normality of pollen grains from partial-sterile plants from PS's mutants. Microsporogenesis from partial-sterile plants was similar to the fertile plants. Microsporogenesis was normal during the course of development resulting in fertile, round pollen grains. The mature pollen grains had vegetative nucleus, generative cell, pollen cell wall and three colpi. The normality of pollen grains from PS's mutants was assessed with different staining reactions and techniques. All techniques indicated that the pollen grains were classified as normal, thus the pollen grains from partial-sterile plants did not differ from pollen grains from fertile plants .

Megagametogenesis of PS-1 showed ovules with unusual orientation with regards to polar nuclei/ secondary endosperm nucleus. The abnormalities, lack of migration, present in the polar nuclei were responsible for the lack of double fertilization, resulting in ovules with embryo but without endosperm. The absence of endosperm was the indirect cause of early embryo abortion observed in the PS-1 mutant. The literature reported others plants in which ovule abortion was due to absence of endosperm (Cichan and Palser, 1982; Arthur

et al., 1993; Vallania et al., 1987; Mogensen, 1982). Megagametogenesis in PS-2, PS-3, and PS-4 showed normal development of partial-sterile plants when compared to the ovule development of fertile plants. The abortion in these mutants was due to lack of double fertilization since the embryo sac was normal at fertilization, presenting egg apparatus and secondary endosperm nucleus.

Aborted ovules showed intact egg apparatus and secondary endosperm nucleus. Lack of fertilization of ovules as an explanation of female sterility has been reported in *Quercus*, cotton and maize (Mogensen, 1975; Contolini and Menzel, 1987; Clark, 1942; Singleton and Mangelsdorf, 1940; Nelson and Clary, 1952; Van Horn and Nelson, 1969)

The importance of this research is based on the observation of the first mutants in soybean presenting female abnormality with complete normality in the male. In soybean the mutations are classified into two types; male-sterile, female-fertile (MS-FF) and male-sterile and female-sterile (MS-FS) (Graybosch and Palmer, 1988). The PS's mutants represent a new class of soybean mutants, male fertile and female partial-sterile. The nontransmission of the partial sterility through the female parent also make these mutants unique in soybean.

The isolation and characterization of mutants identifies the ways in which normal reproductive development can go

wrong, and allows the researcher to dissect the reproductive process and make comparisons with normal development. The mutants provide invaluable raw material for molecular studies (Cove, 1993). According to this author the mutant isolation, genetic analysis of mutants, and phenotypic analysis of mutants are steps necessary to approach the molecular study of the mutants. The current investigation generated results that represent a significant addition to the knowledge of soybean reproductive development. The main objectives, genetic and cytological studies of the PS's mutants, and development comparison with normal (fertile) plants were accomplished.

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